Universitat de Girona

Tesi doctoral

Desenvolupament d'una vacuna contra la mamitis bovina causada per *Streptococcus uberis.*

Rosa Collado Gimbert

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El Dr. Antoni Prenafeta i Amargós, investigador de l'empresa HIPRA SCIENTIFIC S.L.U i acreditat com a director de tesis doctorals per la Universitat de Girona,

DECLARO:

Que aquest treball titulat "Desenvolupament d'una vacuna contra la mamitis bovina causada per *Streptococcus uberis*" que presenta Rosa Collado Gimbert per a l'obtenció del títol de doctora, ha estat realitzat sota la meva direcció.

I, perquè així consti i tingui els efectes oportuns, signo aquest document.

Dr. Antoni Prenafeta Amargós,

Girona, gener 2019

Agraïments

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Índex general

Llista de publicacions derivades de la tesi

Aquesta tesi doctoral es presenta com a un compendi de les següents publicacions, complint els requisits establerts per la Comissió d'Autorització de Defensa de Tesis Doctorals en la sessió del 16 de desembre del 2009.

A continuació es descriuen les referències, àrees temàtiques i factors d'impacte (FI) dels articles publicats:

- Collado R, Montbrau C, March R, Prenafeta A. **Different infection kinetics after an intramammary challenge with *in vitro Streptococcus uberis* biofilm forming and non-biofilm forming strains in dairy cattle.**
  Article sotmès a la revista Veterinary World i en procés de revisió.
  Àrea temàtica: Veterinària
  Índex impacte 2018: 1.12
  Quartil 2018: Segon quartil (Q2)
  SCImago Journal Rank (SJR): 0.454

- Collado R, Prenafeta A, González-González L, Pérez-Pons JA, Sitjà M. **Probing vaccine antigens against bovine mastitis caused by *Streptococcus uberis***. Vaccine. 2016 Jul 19;34(33):3848-54. doi: 10.1016/j.vaccine.2016.05.044.
  Àrea temàtica: Vacunes
  Índex impacte 2016: 3.235
  Quartil 2016: Primer quartil (Q1)

SCImago Journal Rank (SJR): 1.863

- Collado R, Montbrau C, Sitjà M, Prenafeta A. **Study of the efficacy of a *Streptococcus uberis* mastitis vaccine against an experimental intramammary infection with a heterologous strain in dairy cows.** J Dairy Sci. 2018 Nov;101(11):10290-10302. doi: 10.3168/jds.2018-14840.
  Àrea temàtica: *Agriculture, Dairy and Animal Science*
  Índex impacte 2017: 2.749
  Quartil 2017: Primer Quartil Q1
  SCImago Journal Rank (SJR): 1.35

Altres publicacions derivades dels resultats de la present tesi doctoral:

- Collado R, Solà C, Martín R, Roca M, Prenafeta A. (2018). **Analysis of the antibodies induced by UBAC® vaccination.** Pòster a *National Mastitis Conference. Milano 2018*.

- Collado R, Montbrau C, Moreno J, March R, Prenafeta A. (2018). **Efficacy of UBAC® vaccine against an experimental intramammary heterologous challenge in dairy heifers (bacterial count, SCC and serological response).** Pòster a *National Mastitis Conference. Milano 2018.*

- Montbrau C, Collado R, Roca M, Saun X, Prenafeta T, March R. (2018). **Efficacy of UBAC® vaccine against an experimental intramammary heterologous challenge in dairy heifers (clinical response and milk production).** Pòster a *National Mastitis Conference. Milano 2018.*

- Prenafeta, T. Collado, R. (2017). *WO2017140683 (A2*). ***Streptococcus uberis extract as an immunogenic agent.*** European Patent Office. Recuperat de http://www.epo.org/applying/online-services/online-filing.html

Llista d'abreviatures

APC:	Cèl·lules presentadores d'antigen
bFcRn:	de l'anglès, *Bovine neonatal Fc receptor*
cels/mL:	cèl·lules/mil·lilitre
DC:	Cèl·lules dendrítiques
EF-Ts:	Factor d'elongació Ts
EMA:	Agència Europea del Medicament
FBA:	Fructosa bisfosfat aldolasa
GAPDH:	Gliceraldehid-3-fosfat deshidrogenasa
Lf:	Lactoferrina
Ig:	Immunoglobulines
IgG:	Immunoglobulina G
IgG1:	Immunoglobulina G subtipus 1
IgG2:	Immunoglobulina G subtipus 2
IgM:	Immunoglobulina M
IgA:	Immunoglobulina A
IL-1β:	Interleuquina beta 1
IL-8:	Interleuquina 8
IMI:	de l'anglès, *intramamary infection*
LPS:	Lipopolisacàrids
LTA:	Àcid lipoteicoic
Mc:	Macròfags
MEC:	Cèl·lules del teixit epitelial mamari

MHC I:	Complex major d'histocompatibilitat tipus I
MHC II:	Complex major d'histocompatibilitat tipus II
mL:	Mil·lilitre
µM:	Micròmetres
NK:	Cèl·lules natural killer
PAMPs:	Patrons moleculars associats a patògens
PauA:	Activador del plasminògen
PBS:	de l'anglès, *Phosphate buffered saline*
PMN:	Neutròfils polimorfonuclears
PNAG:	poly-N-acetyl-b-(1,6)-glucosamine
PRRs:	Receptors de patrons de reconeixement
$:	Dòlars americans
RCS:	Recompte de cèl·lules somàtiques
SCC:	de l'anglès, *Somatic Cell Count*
SCS:	de l'anglès, *Somatic Cell Score*
SEM:	de l'anglès, *Scanning Electron Microscope*
SUAM:	de l'anglès, *Streptococcus uberis adhesion molecule*
SrtA:	de l'angles, *Sortase A*
UE:	Unió Europea
USA:	United States of America
T_C:	Limfòcits T citotòxics
$T_{H:}$	Limfòcits T helper
TNF-α:	Factor de necrosis tumoral alfa
TLRs:	Toll-like receptors

Tr: Transferrina

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Índex de taules

Resum, Resumen, Abstract

Resum

La mamitis bovina causada per *Streptococcus uberis* és un problema de gran importància en les explotacions lleteres bovines a nivell mundial i en l'actualitat no existeix cap vacuna comercial que hagi demostrat ser eficaç per combatre aquesta patologia. Els objectius de la present tesi doctoral s'emmarquen en les fases 1 i 2 del procés de desenvolupament d'una nova vacuna contra la mamitis bovina causada per *S. uberis*.

El primer objectiu va consistir en el desenvolupament d'un model d'infecció intamamària (IMI) capaç de reproduir la patologia en vaques en lactació, amb la finalitat d'utilitzar-lo per demostrar l'eficàcia dels candidats vacunals obtinguts en aquest treball. Les proves per desenvolupar aquest model d'infecció van començar aconseguint aïllats de casos clínics de mamitis bovina i caracteritzant *in vitro* la seva cinètica de creixement i la seva capacitat de formació de biofilm en placa de 96 pous. En base a aquests resultats inicials, es van seleccionar dues soques i es van infectar experimentalment per via intramamària 2 grups de 4 vaques, amb la mateixa dosi de totes dues soques i en un únic quarteró per vaca. Després de la infecció experimental, es van monitoritzar els signes clínics de mamitis, el recompte de cèl·lules somàtiques (RCS) i el recompte bacteriològic dels quarterons infectats durant 16 dies, així com la producció de llet de les vaques. Els resultats van mostrar diferències clares en la patogenicitat d'ambdues soques, suggerint que la diferent habilitat de formació de biofilm de les soques pot conferir una estratègia de colonització i persistència diferent a la glàndula mamària. Aquests

resultats van servir per seleccionar la dosi i la soca d'infecció per les posteriors proves d'eficàcia en vaques dels candidats vacunals obtinguts en aquesta tesi doctoral.

Per altra banda, degut a la manca d'estudis publicats sobre candidats vacunals eficaços contra la mamitis bovina causada per *S. uberis*, el següent objectiu d'aquesta tesi va consistir en la recerca de nous antígens vacunals. Amb aquesta finalitat, es van seguir dues línies de treball per cercar antígens relacionats amb la formació de biofilm d'*S. uberis*, un factor de virulència que ja havia estat descrit com a rellevant en la mamitis causada per altres patògens. La primera consistia en la identificació de proteïnes immunògenes associades a la paret cel·lular de *S. uberi*s quan aquest creix formant biofilm. La segona línia de treball, es va centrar en l'estudi de components no proteics associats a la formació de biofilm de *S. uberis*.

En la primera aproximació, es va utilitzar un *pool* de sèrums de conill immunitzats amb biofilm de *S. uberis* i un sèrum d'una vaca infectada per *S. uberis*, per detectar proteïnes immunògenes associades a la paret cel·lular bacteriana. Els resultats van permetre identificar un total de 18 proteïnes immunògenes candidates a ser introduïdes en una vacuna. D'aquestes 18 proteïnes, 3 van ser seleccionades per haver estat descrites prèviament com a factors de virulència en altres patògens: la gliceraldehid-3-fosfat deshidrogenasa (GAPDH), la fructosa bisfosfat aldolasa (FBA) i el factor d'elongació Ts (EF-Ts). Posteriorment, diferents grups de ratolins van ser immunitzats amb aquestes proteïnes expressades de manera recombinant i seguidament infectats per via intraperitoneal

amb una soca virulenta de *S. uberis*. Les 3 proteïnes van induir anticossos immunoglobulines G (IgG) en ratolins, però només la FBA i el EF-Ts van reduir la mortalitat dels ratolins després de la infecció experimental, en comparació a un grup control no-vacunat i infectat.

En la segona aproximació, es va treballar en l'extracció i caracterització de components no proteics associats al biofilm de *S. uberis*. El protocol d'extracció es va desenvolupar en base a estudis previs sobre l'extracció de polisacàrids capsulars i exopolisacàrids de la matriu del biofilm de *Staphylococcus aureus* mitjançant un tractament tèrmic. En la caracterització de l'extracte soluble obtingut es va identificar l'àcid lipoteicoic (LTA), descrit a la literatura com un possible factor de virulència de microorganismes Gram positius. En aquesta tesi, per primer cop, es va aconseguir demostrar la seva implicació en la formació de biofilm de *S. uberis in vitro.*

Finalment, com a últim objectiu d'aquest treball, es va avaluar l'eficàcia dels candidats vacunals seleccionats ens les dues aproximacions anteriors, en el model d'IMI en vaques en lactació desenvolupat prèviament. Si bé les proteïnes recombinants FBA i EF-Ts van ser seleccionades per mostrar una eficàcia parcial en el model murí, una vacuna experimental formulada amb les dues proteïnes no va resultar eficaç en front a una infecció experimental en vaques amb una soca virulenta de *S. uberis* (dades no incloses en aquesta tesi). A continuació, es va realitzar un segon estudi d'eficàcia amb una vacuna formulada amb l'LTA extret del biofilm d' *S. uberis*. Aquesta prova va consistir en 2 grups de vaques gestants. El primer grup es

va vacunar amb dues dosis de la vacuna administrades 60 i 21 dies abans del part, i el segon grup amb dues dosis de tampó fosfat salí (PBS) com a grup control. Catorze dies després del part, totes les vaques van ser infectades experimentalment amb una soca virulenta de *S. uberis*. A continuació, es van monitoritzar durant 21 dies els signes clínics de mamitis, el RCS i el recompte bacteriològic dels quarterons infectats, així com la temperatura rectal i la producció de llet de totes les vaques. Els resultats obtinguts van demostrar que, malgrat tots els quarterons infectats van desenvolupar manifestacions clíniques de la patologia, les vaques vacunades van presentar menys signes clínics de mamitis i un menor recompte bacteriològic en els quarterons infectats. A més, després de la infecció experimental, les vaques vacunades van exhibir menor temperatura rectal i menys pèrdues en la producció de llet que les vaques control. Finalment, cal destacar que al final de l'estudi, les vaques vacunades van presentar un major percentatge de quarterons sans, definits com quarterons sense recompte bacteriològic i amb RCS < 200.000 cèl·lules/mil·lilitre (cels/mL), que les vaques control. Així doncs, els resultats d'aquesta prova van confirmar l'eficàcia d'una vacuna experimental basada en un extracte que contenia LTA, davant d'una infecció experimental en vaques en lactació. Per tant, el desenvolupament d'aquesta vacuna va prosseguir, ja fora del marc d'aquesta tesi doctoral, amb els estudis necessaris per complir els requeriments regulatoris pel seu registre i comercialització.

Resumen

La mastitis bovina causada por *Streptococcus uberis* es un problema de gran importancia en las explotaciones bovinas lecheras a nivel mundial y en la actualidad no existe ninguna vacuna comercial que haya demostrado eficacia contra esta patología. Los objetivos de la presente tesis doctoral se enmarcan en las fases 1 y 2 del proceso de desarrollo de una nueva vacuna contra la mastitis bovina causada por *S. uberis*.

El primer objetivo consistió en el desarrollo de un modelo de infección intramamaria (IMI) capaz de reproducir la enfermedad en vacas en lactación, con la finalidad de utilizarlo para demostrar la eficacia de los candidatos vacunales obtenidos como resultado de este trabajo. Las pruebas para desarrollar el modelo de infección empezaron por conseguir aislados de casos clínicos de mastitis bovina y caracterizar su cinética de crecimiento y su capacidad de formar biofilm en placa de 96 pocillos. En base a estos resultados iniciales, se seleccionaron dos cepas y se infectaron experimentalmente por vía intramamaria 2 grupos de 4 vacas en lactación con la misma dosis, en un único cuarterón por vaca. Después de la infección experimental, se monitorizaron los signos clínicos de mastitis, el recuento de células somáticas (RCS) y el recuento bacteriológico de los cuarterones infectados durante 16 días, así como la temperatura rectal y producción de leche de las vacas. Los resultados mostraron diferencias claras en la patogenicidad de las dos cepas, sugiriendo que la diferente habilidad de formar biofilm podía conferir una estrategia de colonización y persistencia diferente

en la glándula mamaria. Estos resultados, sirvieron para seleccionar la dosis y la cepa de infección para posteriores pruebas de eficacia en vacas, con los candidatos vacunales obtenidos en esta tesis doctoral.

Por otra parte, debido a la falta de estudios publicados sobre candidatos vacunales eficaces contra la mastitis bovina causada por *S. uberis*, el siguiente objetivo de la presente tesis doctoral consistió en la búsqueda de nuevos candidatos a antígenos vacunales. Con esta finalidad, se siguieron dos líneas de trabajo para buscar candidatos vacunales relacionados con la formación de biofilm de *S. uberis*, un factor de virulencia que ya había estado descrito como relevante en la mastitis causada por otros patógenos. La primera consistió en la identificación de proteínas inmunogénicas asociadas a la pared celular de *S. uberis* cuando este crece formado biofilm. La segunda línea de trabajo se centró en el estudio de componentes no proteicos asociados a la formación de biofilm de *S. uberis*.

Durante la primera aproximación, se utilizó un *pool* de sueros de conejo inmunizados con biofilm de *S. uberis* y un suero de una vaca infectada por *S. uberis*, para detectar proteínas inmunogénicas asociadas a la pared celular bacteriana. Los resultados permitieron identificar un total de 18 proteínas inmunogénicas candidatas a ser introducidas en una vacuna. De estas 18 proteínas, 3 fueron seleccionadas por haber estado descritas previamente como factor de virulencia en otros patógenos: la gliceraldehido-3-fosfato deshidrogenasa (GAPDH), la fructosa bisfosfato aldolasa (FBA) y el factor de elongación Ts (EF-Ts). Posteriormente, diferentes grupos de ratones fueron inmunizados con estas proteínas expresadas de

manera recombinante y seguidamente infectados por vía intraperitoneal con una cepa virulenta de *S. uberis*. Las 3 proteínas indujeron inmunoglobulinas G (IgG) en ratones, pero sólo FBA y EF-Ts redujeron la mortalidad después de la infección experimental en comparación con un grupo control no vacunado e infectado.

En la segunda aproximación, se trabajó en la extracción y caracterización de los componentes no proteicos asociados al biofilm de *S. uberis*. El protocolo de extracción se desarrolló en base a estudios previos sobre la extracción de polisacáridos capsulares y exopolisacáridos de la matriz del biofilm de *Staphylococcus aureus* mediante un tratamiento térmico. En la caracterización del extracto soluble obtenido, se identificó el ácido lipoteicoico (LTA), descrito en la literatura como un posible factor de virulencia de microorganismos Gram positivos. En la presente tesis, por primera vez, se consiguió demostrar su implicación en la formación de biofilm de *S. uberis in vitro*.

Finalmente, como último objetivo de este trabajo, se valoró la eficacia de los candidatos vacunales seleccionados en las dos aproximaciones anteriores, en el modelo de IMI en vacas en lactación desarrollado previamente. Si bien las proteínas recombinantes FBA y EF-TS fueron seleccionadas por mostrar una eficacia parcial en un modelo murino, una vacuna experimental formulada con las dos proteínas no resultó eficaz frente una infección experimental en vacas con una cepa virulenta de *S. uberis* (datos no incluidos en esta tesis). Por otro lado, se realizó un segundo estudio de eficacia con una vacuna formulada con el LTA obtenido a partir del biofilm de *S.*

uberis. En esta prueba se vacunaron 2 grupos de vacas gestantes. El primer grupo se vacunó con dos dosis de la vacuna administradas 60 y 21 días antes del parto y el segundo grupo con dos dosis de tampón fosfato salino (PBS) como grupo control. Catorce días después del parto, todas las vacas fueron infectadas experimentalmente con una cepa virulenta de *S. uberis*. A continuación, se monitorizaron durante 21 días los signos clínicos de mastitis, el RCS y el recuento bacteriológico de los cuarterones infectados, así como la temperatura rectal y la producción de leche de todas las vacas. Los resultados obtenidos demostraron que, a pesar de que todos los cuarterones desarrollaron manifestaciones clínicas de la patología, las vacas vacunadas presentaron menos signos clínicos de mastitis y un menor recuento bacteriológico en los cuarterones infectados. Además, después de la infección experimental, las vacas vacunadas presentaron menor temperatura rectal y pérdidas de producción de leche que las vacas control. Finalmente, destacar que al final del estudio las vacas vacunadas presentaron un mayor porcentaje de cuarterones sanos, definidos como cuarterones sin recuento bacteriológico y RCS < 200.000 células/mililitro (cels/mL), que las vacas control. Así pues, los resultados de esta prueba confirmaron la eficacia de la vacuna basada en un extracto que contenía LTA, frente a una infección experimental en vacas en lactación. Por lo tanto, el desarrollo de esta vacuna siguió adelante, ya fuera del marco de la presente tesis doctoral, con los estudios necesarios para cumplir los requerimientos regulatorios para su registro y comercialización.

Abstract

Bovine mastitis caused by *Streptococcus uberis* is a major problem in the dairy industry worldwide. Despite of that, there is no vaccine commercial available against it. Therefore, the objectives of this doctoral thesis are framed in phases 1 and 2 of the development process of a new vaccine against bovine mastitis caused by *S. uberis*.

The first objective of this research work was to develop an experimental intramammary infection (IMI) model in dairy cows in order to test experimental vaccines. Two *S. uberis* strains isolated from clinical mastitis cases were selected, one as a biofilm forming strain (SU2H) and the other as a non-biofilm forming strain (0140J) on a polystyrene 96-well microplate assay. Then, two groups of four cows received an intramammary challenge with the same dose of each strain. Following the intramammary challenge, clinical signs of mastitis, milk production, somatic cell count (SCC), rectal temperature and bacterial cell count in milk were recorded daily, for sixteen days post-infection. Results showed that the two strains exhibited clear differences in pathogenicity, suggesting that biofilm formation ability could confer a different colonization and persistence strategy in the mammary gland. Moreover, these results were used to select the strain and dose for further experimental efficacy trials with the vaccine candidates derived from this work.

On the other hand, as a second objective of this thesis, two different approaches were followed in order to detect new virulence factors related with *S. uberis* biofilm, as it has been previously

described as a virulence factor related to bovine mastitis in other pathogens.

The first approach consisted in the evaluation of the potential use as vaccine antigens of some proteins associated to *S. uberis* biofilm, which were previously identified by proteomic and immunological analyses as follows. A pool of serums from rabbits immunized with *S. uberis* biofilm preparations and from a convalescent cow intramammary infected with *S. uberis*, were probed against cell wall proteins from biofilm and planktonic cells previously separated by two-dimensional gel electrophoresis. As a result, 18 immunogenic proteins were identified as putative vaccine candidates. From these proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-biphosphate aldolase (FBA), and elongation factor Ts (EF-Ts) were chosen to be tested as vaccine antigen candidates. For this purpose, different groups of mice were immunized with one of the three recombinant-expressed proteins, and thereafter intraperitoneally challenged with *S. uberis*. The three proteins induced specific IgG antibodies, but a significant reduction of mortality was only observed in the groups of mice vaccinated with FBA or EF-Ts.

The second approach consisted in the extraction and characterization of non-protein components associated to *S. uberis* biofilm. The extraction protocol was developed following previous work on the polysaccharide extraction method of *Staphylococcus aureus*. Then, this extract was characterized and lipoteichoic acid (LTA) was identified. LTA has been previously described as a

virulence factor in other Gram-positive bacterial species, and in the present work, for the first time, it is demonstrated its relationship with the *in vitro* biofilm formation of *S. uberis.*

Finally, as the last objective of this work, the efficacy of the vaccine candidates identified during this thesis was evaluated in the experimental model developed in dairy cows. First, an experimental vaccine was formulated with FBA and EF-Ts, but unfortunately, it did not show to be efficacious against an intramamary challenge with a virulent strain of *S. uberis* (data not shown). Then, the efficacy of an experimental vaccine containing LTA form *S. uberis* was assessed in dairy cows. With this objective, 25 gestating Holstein-Friesian heifers were randomly assigned to one of two groups: group 1 (n = 13), vaccinated by intramuscular route with the vaccine, and group 2 (n =12), vaccinated by intramuscular route with phosphate buffered saline (PBS) as a control group. Both groups were immunized 60 and 21 days before the expected parturition date. Fourteen days after calving all cows were challenged by intramammary infusion of a heterologous *S. uberis* strain in 2 quarters per cow. Then, challenged quarters were monitored for clinical signs of mastitis, bacterial count, and somatic cell count (SCC) for the following 21 days. Rectal temperature and daily milk yield per cow were also assessed. Results showed that all challenged quarters developed clinical mastitis. Nevertheless, vaccination significantly reduced the clinical signs of mastitis, bacterial count, rectal temperature, and daily milk yield losses after the IMI and significantly increased the number of quarters with no bacterial isolation and SCC<200,000 cells/mL at the end of the study. Thus, the results of this trial, confirmed the efficacy

of the vaccine based on an extract containing LTA against an experimental infection in dairy cows. Therefore, the development of this vaccine continued, outside of the context of this doctoral thesis, with the pertinent studies to fulfill the regulatory requirements for its registration and commercialization.

Introducció general

1. Introducció general

1.1. La glàndula mamària bovina

L'espècie bovina presenta quatre glàndules mamàries independents, indistintament anomenades quarterons, íntimament unides entre si i separades per membranes específiques. La unió d'aquestes quatre glàndules mamàries o quarterons forma una unitat específica que s'anomena braguer (Figura 1).

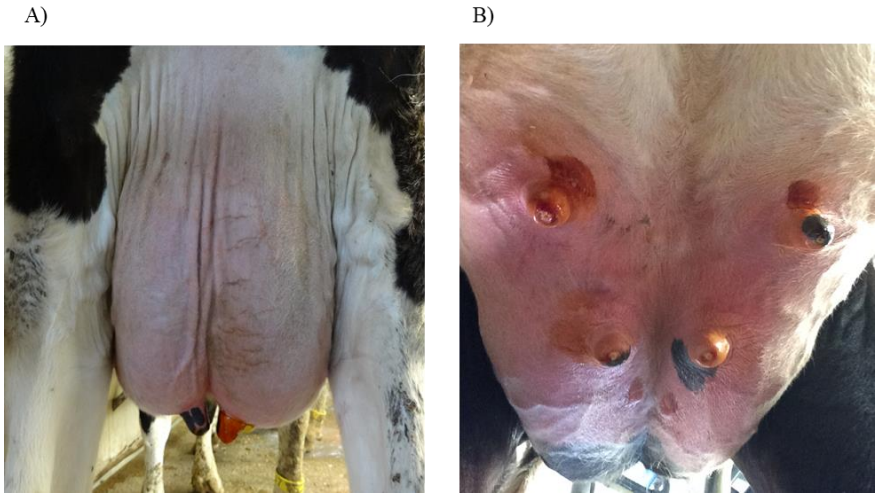

Figura 1. Vista posterior (A) i inferior (B) del braguer d'una vaca en lactació.

1.1.1. Estructura macroscòpica

El braguer és una estructura recoberta per teixit epitelial i lleugerament coberta de pèl. Se situa a la part ventral-inguinal de la vaca i se suporta per l'acció dels lligaments suspensors mitjans i laterals que es troben ancorats als ossos pelvians i als tendons dels músculs abdominals de la zona pelviana. En les vaques el braguer

està dividit en dues meitats (dreta i esquerra) separades pels lligaments suspensors mitjans que ancoren el braguer a la part ventral de la vaca. A cada meitat hi trobem dues glàndules mamàries, una a la part anterior i una altra a la part posterior separades per un septe de teixit connectiu (Figura 2).

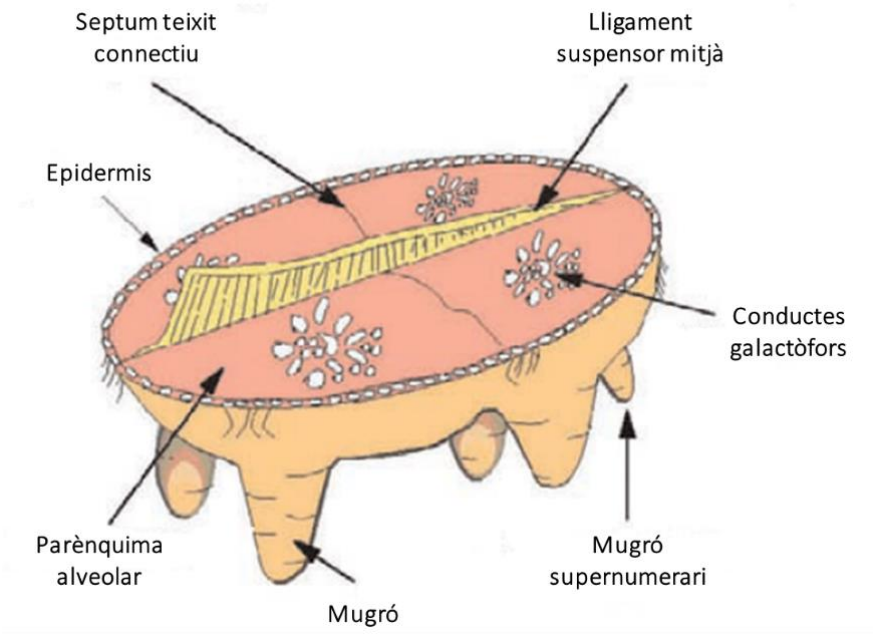

Figura 2. Estructura macroscòpica del braguer. Adaptació de*"Diagrammatic cross section of the four quarters of the udder illustrating the gross anatomy"* de Nickerson i Akers (2012).

Aquesta estructura característica fa que les quatre glàndules mamàries no tinguin connexió directa de dreta a esquera ni de davant a darrera, el que suposa un avantatge en determinades situacions experimentals, ja que a unes glàndules se'ls hi pot administrar un tractament específic, mentre que la resta poden servir com a control. Cal tenir en compte però en els dissenys experimentals, que la

capacitat de les glàndules mamàries posteriors és superior a la de les glàndules mamàries anteriors aproximadament en un 60:40.

Així doncs, les quatre glàndules mamàries funcionen com glàndules separades ja que cadascuna disposa de les seves pròpies estructures i el seu propi parènquima secretor. A més, cada glàndula està directament connectada a la cavitat abdominal pels canals inguinals, a través dels quals, els vasos sanguinis, els vasos limfàtics i els nervis, accedeixen a l'interior del braguer (Nickerson i Akers, 2012).

A la part distal de cada glàndula mamària o quarteró es presenta un mugró desproveït de pèl (Figura 2). Els mugrons són variables en quan a llargada i forma. De manera comú, s'ha estimat que fins en un 40% de les vaques, poden aparèixer mugrons supernumeraris que tendeixen a ser no funcionals. De tota manera, està recomanat eliminar-los ja que poden ser infectats per diferents agents causants de mamitis bovina.

A la part més distal del mugró hi trobem l'esfínter del mugró a través del qual es produeix el buidat de la glàndula mamària (Figura 3). L'esfínter del mugró és la primera línia de defensa en front a les infeccions intramamàries (IMI), també anomenades mamitis. Durant el període sec i entre munyides, aquest roman tancat per acció de la musculatura llisa que envolta el canal del mugró evitant així l'entrada de microorganismes patògens (Sordillo et al., 1997; Rainard i Riollet, 2006). A l'interior, sobre l'esfínter del mugró, hi trobem el canal del mugró que està internament recobert de queratina derivada de

l'epiteli estratificat del canal. La queratina té una doble funció molt important per a evitar noves IMI (Capuco et al., 1992). Per una banda, actua com una barrera física, impedint l'entrada de microorganismes i per l'altra, té una funció bacteriostàtica i bactericida degut a la presència d'agents antimicrobians en la seva composició (Hibbitt et al., 1969; Rainard i Riollet, 2006; Sordillo 2018).

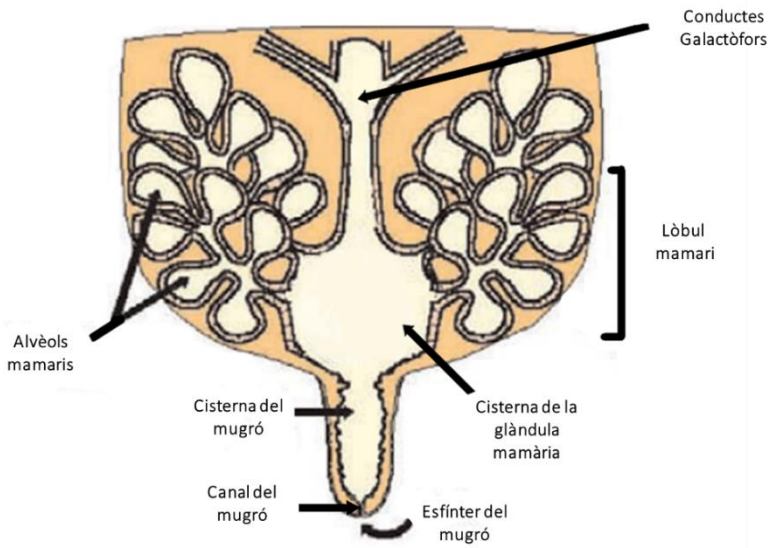

Figura 3. Pla longitudinal de l'estructura de la glàndula mamària. Adaptació de *"Diagram of a mammary quarter illustrating the glandular tissue"* de Nickerson i Akers (2012).

A continuació, trobem la cisterna del mugró, d'uns 5-13 mm de longitud i 0.4-1.63 mm de diàmetre, amb capacitat per emmagatzemar 10-15 ml de llet. Finalment, la cisterna del mugró desemboca a la cisterna de la glàndula mamària amb capacitat per emmagatzemar entre 100 – 2000 ml de llet, de la qual ascendeixen els conductes galactòfors o mamaris que arriben fins als lòbuls

mamaris on es produeix la síntesi de la llet (Figura 3) (Nickerson i Akers, 2012).

1.1.2 Estructura microscòpica

Cada lòbul mamari consisteix en agrupacions de conductes i alvèols mamaris recoberts de teixit connectiu. Els alvèols mamaris, unitat funcional de la glàndula mamària, són els encarregats de la síntesi de llet. Són estructures globulars formades per dos tipus de cèl·lules: les cèl·lules epitelials alveolars i les cèl·lules mioepitelials (Figura 4). Les cèl·lules epitelials alveolars recobreixen l'interior

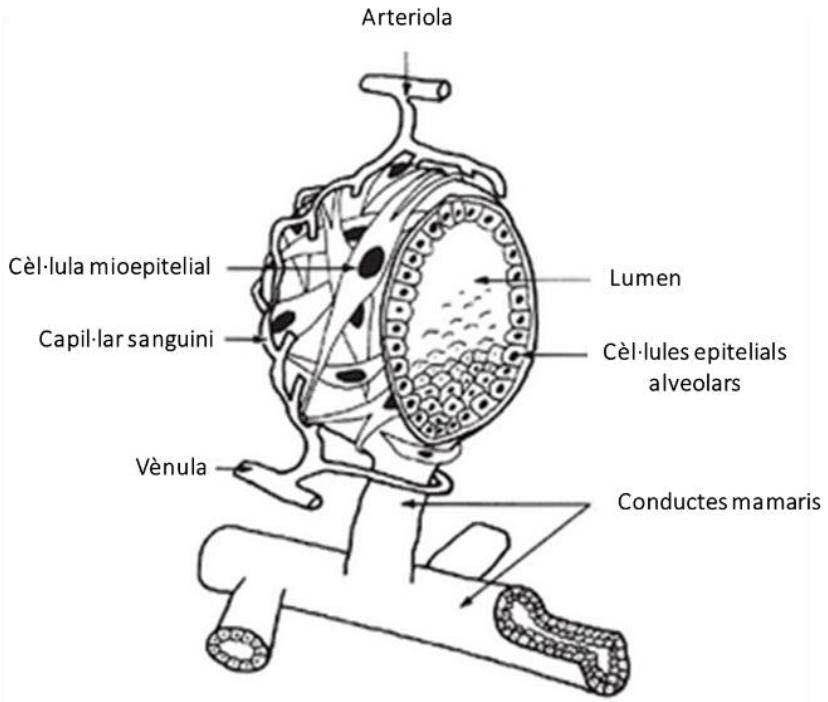

Figura 4. Estructura microscòpica d'un alvèol mamari. Adaptació de *"An alveolus surrounded by blood vessels and myoepithelial cells in the mammary gland"* de Gorden i Timms, (2004).

dels alvèols. Són les encarregades de sintetitzar la llet, a partir de l'absorció de substàncies precursores que provenen dels capil·lars sanguinis adjacents, i secretar-la a la llum de l'alvèol mamari (lumen).

Les cèl·lules mioepitelials, en canvi, es troben a la part externa de l'alvèol i presenten receptors per a l'oxitocina. Aquesta hormona és capaç d'estimular-les i provocar la seva contracció, desencadenant així l'alliberació de la llet des de l'interior de l'alvèol cap als conductes mamaris i fins a l'exterior de la glàndula mamària (Nickerson i Akers, 2012).

1.1.3. Composició de la llet

La llet és la secreció de la glàndula mamària de tots els mamífers i té com a funció principal la nutrició de les seves cries en les primeres etapes de vida. A part de la funció nutritiva, la llet té altres funcions molt importants com la transferència d'immunoglobulines (Ig) i agents antibacterians a la descendència per protegir-la de noves infeccions, la transferència d'enzims i proteïnes per ajudar a la digestió i la transferència d'hormones i factors de creixement essencials per al correcte desenvolupament dels nounats (Fox, 2012b).

Els requeriments nutricionals en cada espècie de mamífer son diferents, i per tant, la composició de la llet es variable entre espècies. A més, la composició de la llet és de naturalesa dinàmica, aquesta varia segons l'etapa de la lactació, l'edat, la raça, l'alimentació, el

balanç energètic i l'estat de la glàndula mamària (Haug, Høstmark, i Harstad, 2007). En la taula 1 és mostra de manera resumida la composició de la llet en diferents espècies de mamífers:

Taula 1. Composició (%) de la llet en diferents espècies de mamífer. Adaptació de *"Composition (%) of the milk of selected species"* (Fox, 2012a)

Espècie	Sòlids totals	Lípids	Proteïna	Lactosa	Cendres
Humana	12,2	3,8	1	7	0,2
Ximpanzé	10,4	1,1	1	8	.
Vaca	12,7	3,7	3,4	4,8	0,7
Búfal	16,8	7,4	3,8	4,8	0,8
Cabra	12,3	4,8	2,9	4,1	0,8
Ovella	19,3	7,4	4,5	4,8	1
Camell	15	5,4	3,8	5,2	0,7
Cavall	11,2	1,9	2,5	6,2	0,5

La llet bovina conté tots els nutrients necessaris pel creixement i desenvolupament dels vedells ja que és una font de lípids, proteïnes (α_1-, α_2-, β- i κ-caseïnes), aminoàcids, vitamines i minerals. Però el seu consum no està limitat només a l'espècie bovina, sinó que és un producte àmpliament consumit per la societat actual. Concretament, en l'actualitat un 85% dels productes làctics i derivats que s'ofereixen al mercat són d'origen boví. No és d'estranyar doncs, el gran interès comercial que hi ha darrera de la producció de llet i que aquesta hagi sigut objecte d'estudi durant els últims 100 anys (Fox, 2012a).

1.1.4 Immunologia de la glàndula mamària

Com hem vist anteriorment, la glàndula mamària disposa d'una sèrie de barreres físiques (esfínter, tap de queratina) com a primera

barrera per protegir la glàndula contra les IMI. Malgrat això, en determinades circumstàncies aquesta primera línia de defensa es pot veure compromesa i s'han d'extremar les mesures d'higiene per evitar noves infeccions. Per exemple, a l'inici de l'eixugat o també anomenat període sec (espai de temps entre el final d'una lactació i el següent part) i just després del part, quan els músculs de l'esfínter es troben més relaxats degut a l'elevada pressió intramamària conseqüència del gran volum de llet retingut, així com després de cada munyida, quan el canal del mugró es manté dilatat durant aproximadament una hora fins tornar-se a tancar (Sordillo, 2018).

El que succeeix quan aquesta primera línia de defensa es veu sobrepassada, és que hi pot haver una entrada de microorganismes a la glàndula mamària, donant lloc a una nova IMI. En aquest punt, l'habilitat de la vaca per resistir l'establiment d'una nova infecció dependrà de l'eficiència del seu sistema immunològic a l'hora de combatre la infecció (Sordillo, 2018).

La resposta del sistema immunològic de la glàndula mamària es pot classificar fonamentalment en dues categories, la resposta immune innata, que s'activa immediatament després de l'entrada del microorganisme patogen, i la resposta immune adaptativa, que requereix d'hores o dies per la seva activació, però que en contrapartida és molt més específica contra el patogen (Sordillo, 2018). Cal destacar però, que es necessària la correcta activació i interacció de tots dos tipus de resposta per obtenir la protecció necessària contra els microorganismes invasors, per una banda

evitant la invasió i colonització dels teixits mamaris i per l'altra per restablir el correcte funcionament de la glàndula mamària un cop passada la infecció (Aitken, Corl, i Sordillo, 2011).

1.1.4.1. Resposta immune innata de la glàndula mamària

La resposta immune innata és doncs la primera línia de defensa a nivell immunològic quan la glàndula mamària es envaïda per un agent causant de mamitis. Aquesta resposta immune innata es composa de diferents mecanismes efectors no específics de patogen, alguns dels quals es resumeixen en la taula 2.

Per una banda tenim les barreres físiques com son l'esfínter de la glàndula mamària i el recobriment de queratina del canal del mugró. La queratina té doble acció, per una banda actua com a barrera física, i per l'altre com a agent bacteriostàtic i bactericida, degut als àcids grassos i molècules antimicrobianes que composen la seva part lipídica (Sordillo, 2018).

A continuació, també trobem una altra línia de defensa formada per proteïnes i enzims de la llet amb acció bactericida i bacteriostàtica, com per exemple la Lactoferrina (Lf), que actua com a quelant del ferro disponible en el medi. Així doncs, els microorganismes amb requeriments més elevats de ferro per al seu creixement, seran més susceptibles a l'efecte de la Lf, com per exemple *Escherichia coli* i *Staphylococcus aureus* (Legrand, Elass, Pierce, i Mazurier, 2004; Rainard, 1986).

Taula 2. Mecanismes efectors de la resposta immune innata a la glàndula mamària.

Tipus	Mecanisme	Funció principal	Font
Barrera física	Esfínter del mugró	Evitar la penetració de bacteris patògens a l'interior del canal del mugró.	(Rainard i Riollet, 2006; Sordillo, 2018)
	Canal del mugró	Síntesi de queratina com a barrera física per l'entrada de patògens alhora amb activitat bacteriostàtica.	(Rainard i Riollet, 2006; Sordillo, 2018)
Proteïnes	Complement	Facilitar als PMN opsonització i fagocitosi dels bacteris. Lisi bacteriana	(Rainard, 2003; Sordillo, 2018)
	Lactoferrina (Lf)	Quelant de ferro amb acció bacteriostàtica i bactericida.	(Rainard i Riollet, 2006)
	Transferrina (Tr)	Quelant de ferro amb acció bacteriostàtica.	(Rainard i Riollet, 2006)
Enzims	Lisozim	Acció bactericida actuant de manera sinèrgica amb anticossos, complement o lactoferrina	(Rainard i Riollet, 2006)
	Lactoperoxidasa	Acció bactericida en presència de peròxid d'hidrogen.	(Rainard i Riollet, 2006)
	Xantina oxidasa	Acció bactericida per la generació de molècules reactives del oxigen.	(Rainard i Riollet, 2006)

Tipus	Mecanisme	Funció principal	Font
Anticossos	Anticossos no específics	Facilitar opsonització i fagocitosi	(Rainard i Riollet, 2006)
PRRs	Toll-like receptors (TLRs)	Reconeixement de patògens i activació de la resposta inflamatòria	(Li i Verma, 2002; Goldammer et al., 2004)
Cèl·lules	Neutròfils polimorfonuclears (PMN)	Fagocitosi bacteriana, acció bactericida, formació d'una trampa extracel·lular rica en substàncies antimicrobianes (NET).	(Lippolis, Reinhardt, Goff, i Horst, 2006; Paape, Shafer-Weaver, Capuco, Van Oostveldt, i Burvenich, 2002; Sordillo, 2018)
	Macròfags (Mc)	Fagocitosi bacteriana, producció de citoquines proinflamatòries, presentació antigen via MHC II	(Fitzpatrick et al., 1992; Politis et al., 1992; Sordillo, 2005; Rainard i Riollet, 2006)
	Cèl·lules Natural Killer (NK)	Eliminació dels patògens intracel·lulars	(Rainard i Riollet, 2006; Sordillo, 2018)
	Cèl·lules dendrítiques (DC)	Fagocitosi bacteriana i presentació antígens a limfòcits via MHC I MHC II	(Sordillo, 2018)

Per altra banda, dins dels mecanismes efectors de la immunitat innata hi incloem també els receptors de reconeixement de patrons (PRRs, per les seves sigles en anglès, de *Pattern Recognition Receptor*). Aquests receptors són presents tant a les cèl·lules del sistema immunològic com a les cèl·lules epitelials de la glàndula mamària i tenen un paper molt important en el reconeixement de patrons moleculars conservats i específics entre determinats grups de patògens, els anomenats PAMPs, per les seves sigles en anglès de *Pathogen-Associated Molecular Patterns*. Específicament a les cèl·lules del teixit epitelial mamari (MEC), el reconeixement d'aquests PAMPs per part dels PRRs activa la secreció de quimoquines com IL-8 que contribueixen al reclutament de neutròfils polimorfonuclears (PMN) a la llum de la glàndula mamària (Alnakip et al., 2014). Alguns exemples de receptors PRRs son els Toll-like receptors (TLRs), una família de proteïnes transmembrana expressades per les cèl·lules epitelials, els leucòcits i les cèl·lules endotelials (Jungi, Farhat, Burgener, i Werling, 2011; Kumar, Kawai, i Akira, 2011). En el cas de la mamitis bovina són d'especial importància el TLR-2 i TLR-4 ja que reconeixen PAMPs associats a microorganismes causants de mamitis bovina, com peptidoglicans i lipopolisacàrids (LPS) respectivament (Goldammer et al., 2004). El reconeixement d'aquests PAMPs per part dels TLRs, desencadena l'activació de diferents cascades metabòliques intracel·lulars, com la transcripció del factor nuclear NF-kβ que és un modulador tant de la resposta immune innata com l'adquirida (Li i Verma, 2002).

Finalment, la glàndula mamària també disposa d'una línia de defensa cel·lular composada majoritàriament per PMN, macròfags (Mc), cèl·lules natural killer (NK) i cèl·lules dendrítiques (DC) (Rainard i Riollet, 2006). En una glàndula mamària sana, la concentració de cèl·lules esperada s'estableix entre 100.000 i 200.000 RCS/mL de llet, i recomptes superiors s'associen a infeccions intramamàries ja siguin de tipus clínic o subclínic (Harmon, 1994).

La tipologia cel·lular majoritària en la llet i els teixits d'una glàndula mamària sana (involucionada o en lactació) són els Mc (Sordillo, 2018). Quan es desencadena una IMI, la funció d'aquests Mc és la de fagocitar els patògens invasors i alhora sintetitzar citoquines proinflamatòries per iniciar la resposta inflamatòria, com la interleuquina beta 1 (IL-1β) i el factor de necrosi tumoral alfa (TNF-α). Aquestes citoquines contribueixen a la migració dels PMN, des de la circulació sanguínia a la llet, i estimulen la seva activitat bactericida (Rainard i Riollet, 2003). D'altra banda, els Mc també juguen un paper important en l'inici de la resposta immune adaptativa, esdevenint cèl·lules presentadores d'antigen (APC) via el complex major d'histocompatibilitat tipus II (MHC II) (Fitzpatrick et al., 1992; Politis et al., 1992).

Durant la fase infamatòria, els PMN esdevenen la tipologia cel·lular principal a la glàndula mamària. La seva funció és principalment la de fagocitar els agents invasors, i la rapidesa i la magnitud de la seva migració des del torrent sanguini fins a la glàndula mamària són factors determinants en el curs de la infecció

(Paape et al., 2002). La migració de PMN està regulada per l'expressió de citoquines proinflamatòries com la interleuquina 8 (IL-8), que s'expressa en el moment que les MEC entren en contacte amb un bacteri que vol colonitzar l'epiteli (Figura 5) (Rainard i Riollet, 2003).

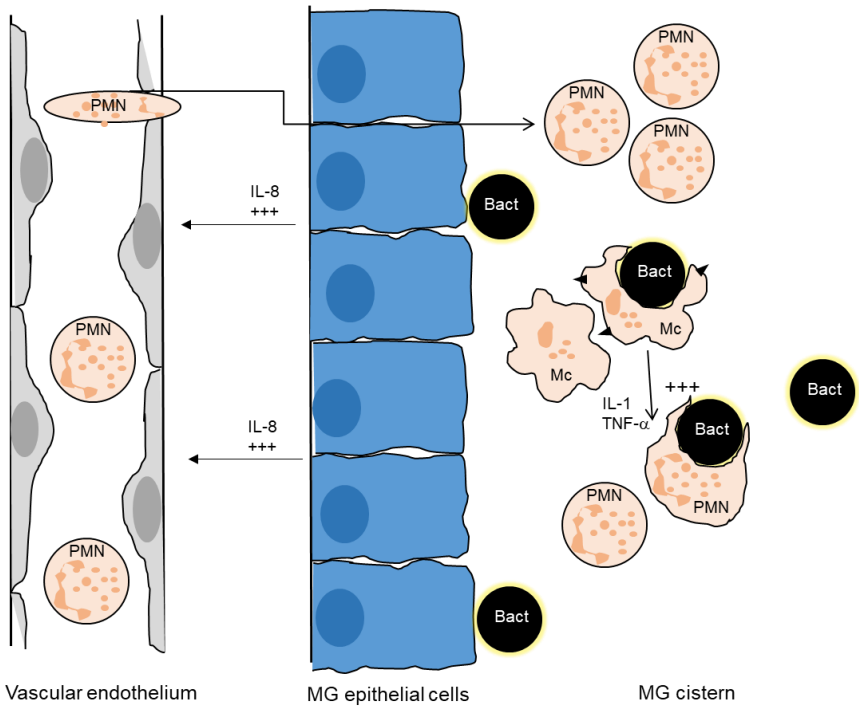

Figura 5. Esquema de la migració i l'activació de PMN a la glàndula mamària.

L'expressió de IL-8 per part de les MEC, genera un gradient fins a l'endoteli vascular que indueix l'adhesió dels PMN a aquest i la seva posterior extravasació fins a l'epiteli mamari (Lee i Zhao, 2000). Per alta banda, l'estimulació dels Mc sembla tenir també un paper important en el reclutament de PMN (Craven, 1983), atribuïble a la síntesi de citoquines proinflamatòries com IL-1 i TNF-α (Sordillo i Streicher, 2002; Sordillo, 2005).

Cal destacar que la funció fagocítica dels PMN es pot veure incrementada substancialment en presència d'anticossos opsonitzants i /o en presència de l'acció de les proteïnes del complement (Paape et al., 2002; Rainard i Riollet, 2006). Alguns autors com Lippolis i els seus col·laboradors (2006) han descrit també com, a part de la funció fagocítica, els neutròfils són capaços de formar una trampa extracel·lular, en anglès *neutrophil extracellullar trap* (NET), com a mecanisme addicional de defensa en front als patògens. Aquest mecanisme consisteix en l'alliberació de material nuclear, proteïnes granulars i fibres extracel·lulars per part dels neutròfils, en resposta a la interacció d'aquests amb un patogen. Aquest material formaria una trampa on quedarien retinguts els patògens i podrien ser eliminats per una via independent a la fagocitosi.

1.1.4.2. Resposta immune adaptativa de la glàndula mamària

Al contrari del que succeeix amb la resposta immune innata, la resposta immune adaptativa és capaç de dirigir una resposta específica contra un determinat microorganisme patogen. A més, aquesta resposta és capaç de generar una memòria immunològica que perdurarà en la vaca fent que en una propera exposició al mateix patogen l'animal reaccioni de manera més ràpida i més efectiva contra aquest (Sordillo, 2018).

Els principals tipus cel·lulars de la resposta immune adaptativa són les cèl·lules APC, que inicien la resposta immune adaptativa, i els limfòcits B i T, que són els principals efectors de la resposta. Les

principals APC són les DC, que ingereixen el patogen a la llum de la glàndula mamària, maduren i migren als ganglis limfàtics supramamaris on presentaran l'antigen als limfòcits T *naive*. Altres cèl·lules amb capacitat de presentació d'antigen són els macròfags i els limfòcits B (Sordillo, 2018).

L'estimulació dels limfòcits T per part de les cèl·lules APC resultarà en la seva proliferació, donant lloc a diferents subpoblacions de cèl·lules efectores que variaran en funció de l'estímul antigènic rebut: els limfòcits γδ i els limfòcits αβ, que es subdivideixen en: els limfòcits T citotòxics CD8+ (Tc) que reconeixen els antígens exposats pel complex major d'histocompatibilitat tipus I (MHC I) i limfòcits T *helper* CD4+ (T_H) que reconeixen els antígens associats a MHC de classe II. Al mateix temps, existeixen diferents subpoblacions de limfòcits T *helper* CD4+: Th1, Th2, Th17 i els T *reguladors* (Treg) en funció de les citocines que expressen.

La funció més important dels limfòcits T *helper* dins de la resposta immune adaptativa, és l'activació dels limfòcits B, que al mateix temps podran esdevenir APC, cèl·lules de memòria o cèl·lules plasmàtiques secretores d'anticossos o Ig específiques (Sordillo, 2018; Sordillo et al., 1997).

Les Ig són els efectors solubles principals de la resposta immune adaptativa. Existeixen 4 classes d'Ig específiques a la glàndula mamària que juguen diferents rols davant la invasió per un patogen: la immunoglobulina G_1 (IgG_1), la immunoglobulina G_2 (IgG_2), la immunoglobulina M (IgM), i la immunoglobulina A (IgA) (Taula 3).

Taula 3. Immunoglobulines específiques presents a la llet de la glàndula mamària. Adaptació de *"The role of immunoglobulins in mammary gland defense"* de Sordillo i Aitken, 2012.

Isotip	Funció biològica
IgG_1	Opsonitzar bacteris per optimitzar la fagocitosi per part dels Mc.
IgG_2	Opsonitzar bacteris per optimitzar la fagocitosi per part dels PMN i Mc.
IgA	Associada a la porció lipídica de la llet. No uneix complement ni té capacitat opsonitzant. Aglutinació, neutralització de toxines i interferència adhesió bacteriana.
IgM	Fixació del complement, opsonització, aglutinació i neutralització de toxines. Funció opsonitzant en presència del complement.

La funció principal de les IgG_1, IgG_2 i IgM és actuar com a opsonines i facilitar l'activitat fagocítica de PMN i Mc (Korhonen, Marnila, i Gill, 2000; Paape et al., 2002), mentre que les IgA tenen la funció de neutralitzar toxines i provocar l'aglutinació dels bacteris interferint en la disseminació del patogen i la colonització de la glàndula mamària (Newby i Bourne, 1977; Korhonen et al., 2000).

La concentració de cada classe de Ig en llet és depenent de l'estadi de lactació en el qual es trobi la vaca i de l'estat de la glàndula mamària. En una glàndula sana, la IgG més abundant és la IgG_1 tant en llet com a calostre (Newby i Bourne, 1977; Barrington et al., 1997), mentre que durant la fase inflamatòria, la IgG_2 és la que incrementa de manera substancial (Korhonen et al., 2000). Aquestes IgG són secretades a nivell local per limfòcits B que s'han activat i

han proliferat donant lloc a cèl·lules plasmàtiques, o bé son transportades específicament des del torrent sanguini fins a la llum de la glàndula mamària, gràcies a l'existència de receptors específics a les MEC com el receptor bFcRn, de l'anglès, *bovine neonatal Fc receptor* (Cui et al., 2014).

1.2. Mamitis bovina

Es defineix mamitis bovina com la reacció inflamatòria de la glàndula mamària en resposta a una IMI per un microorganisme, generalment d'origen bacterià. En funció de l'agent etiològic les mamitis es poden dividir en mamitis contagioses i ambientals, i segons el quadre clínic, en clíniques i subclíniques.

1.2.1. Classificació segons els agents causants. Mamitis contagioses i ambientals.

Els agents causals de mamitis es divideixen en dos grans grups: els agents contagiosos i els ambientals.

Els microorganismes contagiosos són aquells que tenen com a reservori principal el propi animal, la vaca, i la transmissió dels quals es produeix per contacte directe amb material infectat, com poden ser altres glàndules mamaries infectades, aparells de munyir contaminats etc. Generalment aquests patògens, s'associen a infeccions de tipus crònic o subclínic (Biggs, 2009). Durant dècades, *Staphylococcus aureus* i *Streptococcus agalactie* han estat considerats els microorganismes causants de mamitis contagioses més importants (Ruegg, 2017).

En canvi, els microorganismes classificats com ambientals, són aquells capaços de sobreviure en l'ambient, especialment en zones humides i amb una alt contingut en matèria orgànica. Aquestes infeccions, a diferència de les causades per microorganismes contagiosos, es poden donar tant durant el període de lactació com en període sec, degut a la capacitat d'aquests microorganismes ambientals per sobreviure i multiplicar-se tan dins com a fora de la glàndula mamària. Els microorganismes patògens classificats com ambientals de manera més comú són les espècies incloses dins el grup de coliforms, com *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytocia, Enterobacter aerogenes, Citrobacter spp;* altres espècies de Gram negatius com *Serratia spp i Proteus spp*, i diferents espècies *d'Streptococcus,* entre les quals destaca com a principal *S. uberis* (Smith et al., 1985).

1.2.2. Classificació segons els signes clínics. Mamitis cliniques i subcliniques.

Parlem d'una mamitis clínica quan a conseqüència de la IMI s'observen alteracions macroscòpiques a la llet i/o apareixen signes clínics al braguer com inflamació, enduriment, envermelliment i dolor al tacte. La llet presenta un aspecte i composició alterada, similar a la del sèrum sanguini amb o sense presència de coàguls (Figura 6), i amb un RCS de l'ordre 2.000.000 cèl·lules/mil·lilitre (cels/mL).

Aquestes cèl·lules somàtiques són majoritàriament PMN, que han migrat des del torrent sanguini fins a la glàndula mamària com a resposta a la inflamació generada davant de la IMI (Leigh, 1999). En els casos més severs, una mamitis clínica pot conduir també a un increment sistèmic de la temperatura de la vaca i/o una infecció generalitzada que pot desembocar en la mort de l'animal (Leigh, 1999).

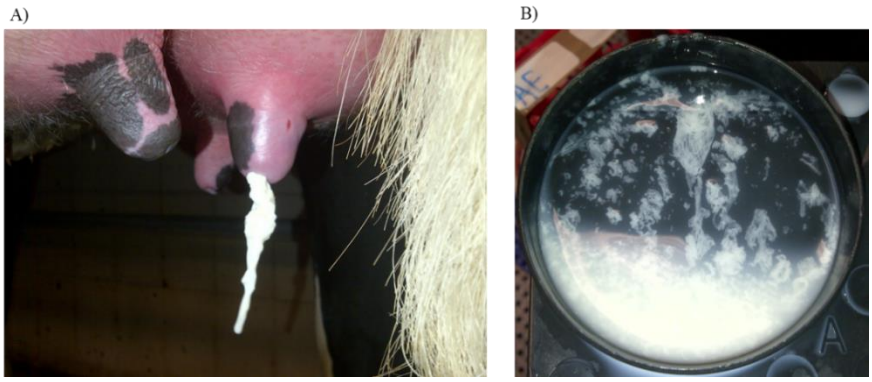

Figura 6. (A) Envermelliment i enduriment de la part distal de la glàndula mamària en un cas de mamitis clínica. (B) Alteracions macroscòpiques de la llet en un cas de mamitis clínica.

En el cas d'una mamitis subclínica en canvi, la infecció no causa alteracions macroscòpiques en la llet ni en els quarterons afectats, però si que existeix també un increment en el recompte de RCS, generalment superior a 250.000 cels/mL de llet (Leigh, 1999).

Així doncs, una IMI, ja cursi de manera clínica o subclínica, sempre resultarà en un increment dels RCS i en una disminució de la quantitat i la qualitat de la llet.

1.2.3. Repercussions Econòmiques

La mamitis bovina està considerada com la patologia més freqüent i amb majors repercussions econòmiques en la indústria lletera bovina. S'ha estimat que la taxa d'incidència de mamitis clínica va de 13 a 40 casos per cada 100 vaques i any en diferents països i sistemes de maneig (Jamali et al., 2018). Aquest elevat impacte econòmic està associat fonamentalment a dos factors: les pèrdues directes i indirectes en la producció i qualitat de la llet; i les despeses en les mesures de control de les IMI, com són l'ús de tractaments específics, costos veterinaris i el sacrifici prematur de les vaques permanentment infectades (Seegers et al., 2003).

Alguns autors com Rollin i col·laboradors (2015) han estimat en 444 dòlars americans ($) el cost d'un cas de mamitis clínica durant els primers 30 dies de lactació. Les pèrdues directes inclouen el diagnòstic (10$), el tractament (36$), la llet descartada degut als residus d'antibiòtics (25$), els serveis veterinaris (4$), la feina associada (21$) i la pèrdua d'animals (32$). Les pèrdues indirectes en canvi, inclouen la pèrdua de llet que tindrà una vaca que ja ha patit un cas de mamitis (125$), els sacrificis prematurs dels animals (125$) i les possibles alteracions reproductives (9 $).

Les pèrdues econòmiques associades a mamitis subclíniques en canvi, són molt més difícils de calcular, ja que el seu diagnòstic sovint passa desapercebut (Rollin, Dhuyvetter, i Overton, 2015; Ruegg, 2017). En l'actualitat, alguns autors descriuen que les mamitis d'origen subclínic són les que generen pèrdues econòmiques més importants a les explotacions com a conseqüència de la

disminució de la producció i de la qualitat de la llet. (Zadoks i Fitzpatrick, 2008).

En l'actualitat les pèrdues econòmiques es calculen mitjançant dos indicadors: el *score* de cèl·lules somàtiques (SCS) (Ali i Shook, 1980; Wiggans i Shook, 1987) que determina la relació lineal entre la incidència de mamitis subclíniques i la disminució en la producció de llet; i per altra banda, el RCS a tanc, en funció del qual es determina la qualitat de la llet de l'explotació i el seu valor econòmic. (Leigh, 1999; Nightingale, Dhuyvetter, Mitchell, i Schukken, 2008).

1.2.4. Mesures de control i situació actual

La mamitis bovina és una patologia multifactorial que requereix de la combinació de múltiples factors per a que es desencadeni: exposició al patogen, condicions ambientals adequades, balanç energètic de l'animal etc. i presenta una resposta molt variable en funció de l'animal infectat (McDougall, Parker, Heuer, i Compton, 2009). Així doncs, davant d'una patologia tan complexa, la correcta identificació i estudi de totes les variables implicades és l'única manera de dissenyar bones estratègies de prevenció i control.

Amb l'objectiu de minimitzar l'exposició al patogen, ja fa més de 25 anys que a les explotacions bovines lleteres s'ha implementat el *"five-point contol plan"*. Aquesta estratègia té origen en els primers estudis de Neave et al (1969), on es va observar que amb la implementació de les mesures d'higiene adequades abans, després i durant la munyida, es reduïa significativament l'exposició als patògens i com a conseqüència hi havia una disminució en les taxes

de noves infeccions. Posteriorment, en base a aquests estudis, es va desenvolupar el *five-point control plan* tal i com es coneix en l'actualitat i que consisteix en: 1- un tractament desinfectant dels mugrons abans i després de la munyida, 2- l'ús d'antibiòtic en el moment de l'eixugat al final de cada lactació, 3- el tractament adequat de les vaques amb casos clínics, 4- el sacrifici de les vaques persistentment infectades i 5- el manteniment i desinfecció adequats del sistema de munyida (Ruegg, 2017). La implementació d'aquest pla durant les últimes dues dècades ha reduït significativament les noves IMI d'origen contagiós, però malauradament és un nínxol que han ocupat els patògens ambientals (Leigh et al., 1999), de manera que podem dir que encara queda molt camí per recórrer en el camp de la prevenció i control de la mamitis.

Per altra banda, l'aplicació d'antibiòtics de manera sistemàtica tan de forma preventiva a l'inici del període d'eixugat, com en forma de tractament durant la lactació, pot portar associats problemes molt greus a nivell de salut pública. L'aparició de residus d'antibiòtics en llet i el potencial efecte que aquest ús sistemàtic pot tenir en l'aparició de resistències és quelcom que cada cop més preocupa als consumidors i les autoritats. Així doncs, cal estudiar més a fons com aplicar l'ús d'antibiòtics sense comprometre la salut pública (Ruegg, 2017) i per altra banda trobar altres mesures preventives més segures com la vacunació dels animals.

Finalment, alguns autors estan estudiant altres tècniques per reduir la incidència de mamitis, com la selecció genètica d'animals resistents a noves infeccions, així com també la suplementació de la

dieta amb nutrients com vitamina E i el seleni, que han demostrat ser essencials pel correcte funcionament de la glàndula mamària i la funció fagocítica dels neutròfils (Ruegg, 2017).

1.2.4.1. Vacunació com a estratègia de prevenció

En vista de les limitades mesures de control contra les mamitis de les que es disposa actualment, no cal dir que el desenvolupament de vacunes eficaces per a protegir les vaques contra les noves IMI és una fita molt important per a la industria lletera (Ruegg, 2017). Cal destacar però que, malgrat que l'ús de vacunes en el control d'altres patologies és molt efectiu, en el camp de la mamitis ha sigut un gran repte fins a dia d'avui aconseguir vacunes eficaces per la naturalesa multifactorial d'aquesta patologia.

Les vacunes contra la mamitis bovina basen la seva eficàcia en l'estimulació d'una resposta immune adaptativa contra determinants antigènics específics. D'aquesta manera, quan l'animal entra en contacte amb una patogen en concret, s'estimula de manera ràpida la resposta cel·lular adaptativa donant lloc a la producció d'anticossos específics contra el patogen en sèrum i en llet (Sordillo, 2018).

En l'actualitat existeixen diferents vacunes al mercat per a la prevenció de les mamitis contagioses i ambientals causades per *E. coli, S. aureus i Klebsiella spp.* (Taula 4). Cal destacar també la existència d'una vacuna contra la mamitis bovina causada per *S. uberis*, però a diferencia de la resta, només està registrada a USA amb una llicència condicional, el que significa que és un producte sobre el qual no hi ha proves d'eficàcia preclíniques ni clíniques publicades.

Taula 4. Vacunes comercials disponibles contra la mamitis bovina.

Nom comercial	Tipus de vacuna	Companyia	Mercat
ENVIRACOR™ J-5	Bacterina *Escherichia coli* J5	Zoetis	USA
J-VAC®	Bacterina-Toxoide *Escherichia coli* J5	Merial	USA
Lysigin®	*Staphylococcus aureus*	Boehringer Ingelheim	USA
Startvac®	Bacterina *Escherichia coli* J5 i *Staphylococcus aureus*	HIPRA	64 països
KLEBVax™	Vacuna contra la mamitis causada per *Klebsiella spp*.	Agrilabs	USA
Streptococcus Uberis* Bacterin	Bacterina *Streptococcus uberis*	Hygieia	USA
UBAC®**	Subunitat contra *Streptococcus uberis*	HIPRA	UE

*Vacuna amb llicència condicional a USA, sense estudis eficàcia publicats.

**Vacuna desenvolupada com a resultat d'aquesta tesi doctoral.

1.3. Streptococcus uberis

1.3.1. Característiques generals

Streptococcus uberis és un coc Gram positiu, catalasa positiu, oxidasa negatiu, d'uns 0,5-1 micròmetres (µM) de diàmetre que s'ha convertit un dels principals agents etiològics de mamitis clínica i subclínica de la industria lletera bovina mundial (Figura 7).

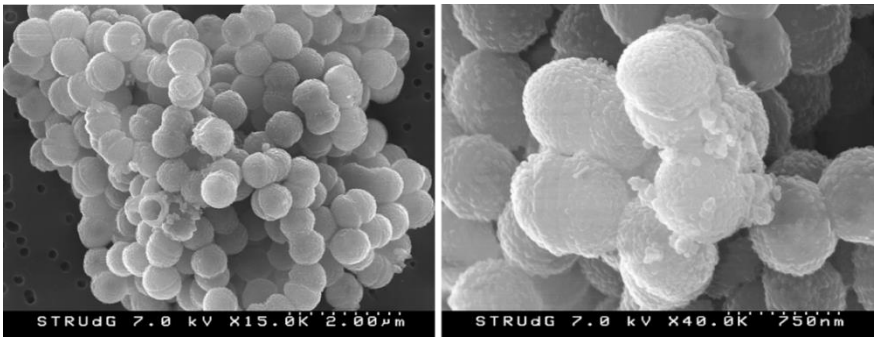

Figura 7. Imatges de microscòpia electrònica de rastreig (SEM) d'una soca de *S. uberis* aïllada d'un cas clínic de mamitis bovina.

Les IMI causades per *S. uberis* desencadenen tant casos clínics com subclínics (Koivula, Pitkälä, Pyaörälä, i Mäntysaari, 2007). En països com Canadà, els Estats Units i Holanda *S. uberis* és responsable del 14 - 26% dels casos de mamitis clínica. A UK s'ha descrit com el principal agent de mamitis clínica (Bradley, Leach, Breen, Green, i Green, 2007), i en països com Nova Zelanda i Austràlia, on basen el seu sistema productiu en la pastura, és el principal agent causant de mamitis clínica (Hogan i Smith, 1997; Wang et al., 1999; McDougall et al., 2007).

A la península ibèrica, la incidència de IMI causades per *S. uberis*, respecte el total de casos de mastitis, presenta una distribució

variable (Timón i Jiménez, 2006): 13,8% a Galícia, 13,5% a Astúries, 17,4% a Navarra i País Basc, 8,4% a Castella i Lleó, 11,2% a Castella-La Manxa i Portugal, 16,3% a Andalusia i un 5,5% a Catalunya.

1.3.2. Epidemiologia

Històricament, l'espècie *S. uberis* estava dividida en 2 serotips, el serotip I i serotip II. Tots dos serotips s'havien aïllat de casos clínics de mamitis bovina, però amb els anys es va descobrir que el serotip II de *S. uberis* es corresponia amb una espècie bacteriana diferent que avui en dia coneixem com *Streptococcus parauberis* (Williams i Collins, 1990). Des de llavors, molts autors han intentat identificar i classificar en grups patogènics diferents aïllats de *S. uberis* mitjançant diverses de tècniques de tipificació molecular (Archer, Bradley, Cooper, Davies, i Green, 2017; Jayarao, Oliver, Tagg, i Matthews, 1991; Phuektes et al., 2001). Fins al moment però, no s'han identificat genotips ni serotips que puguin estar associats a un tipus d'IMI específica.

Tot i així, alguns estudis si que han aconseguit demostrar que malgrat que a les explotacions lleteres hi ha una alta diversitat de soques de *S. uberis* capaces de causar mamitis (Phuektes et al., 2001), algunes soques són identificades de manera majoritària en els animals d'una mateixa granja (Archer et al., 2017; Davies et al., 2016; Zadoks et al., 2003). Això els condueix a pensar que *S. uberis*, històricament classificat com un patogen ambiental, en certes ocasions pot tenir un comportament contagiós i transmetre's d'animal en animal. En aquest sentit, *S. uberis* presentaria dos grups diferenciats de soques:

les soques adaptades, capaces de propagar-se de vaca a vaca per contagi i donar lloc a infeccions persistents, i les no adaptades, aquelles que s'adquireixen de l'ambient i resulten en infeccions transitòries de curta durada. (Tassi et al., 2013).

1.3.3. Factors de virulència

Existeixen diversos treballs que descriuen possibles factors de virulència associats a les IMI causades per *S. uberis*. De tota manera, en la majoria de casos, el seu mecanisme d'acció no és clar. En la taula número 5 es mostra un resum dels principals factors de virulència descrits a la literatura.

Taula 5. Principals factors de virulència descrits a la literatura per *S. uberis.*

Factor de virulència	Funció	Font
Càpsula	Antifagocítica.	(Almeida i Oliver, 1993)
LTA	Adhesió a les cèl·lules MEC.	(Almeida et al., 1996)
Proteïna M 24	Adhesió a les cèl·lules MEC.	(Almeida et al., 1996)
PauA	Utilització de nutrients.	(Leigh et al., 1999)
MtuA	Captació del magnesi durant el creixement bacterià.	(Smith et al., 2003)
SrtA	Ancoratge de proteïnes a la paret cel·lular.	(Leigh, Egan, Ward, Field, i Coffey, 2010)
SUAM	Adhesió a les cèl·lules MEC.	(Almeida, Luther, Park, i Oliver, 2006)
Uberis factor	Factor co-hemolític	(Skalka et al., 1980)

Entre els factors de virulència descrits, hi trobem factors antifagocítics com la càpsula; proteïnes implicades en l'obtenció de nutrients i creixement bacterià, com l'activador del plasminogen (PauA) i molècules involucrades en l'adhesió de *S. uberis* a les MEC com l'àcid lipoteicoic (LTA) i la proteïna SUAM (de l'anglès, *S. uberis adhesion molecule*).

Per altra banda, en un intent de descriure nous factors de virulència Ward i col·laboradors (2009) van seqüenciar el genoma de la soca *S. uberis* 0140J. A partir d'aquí van elaborar una llista de gens que podrien estar relacionats amb factors de virulència*:* SUB1111 (Fibronectin-binding protein), SUB1273 (Hemolysin like protein), SUB1154 (C5a peptidase precursor), SUB0881 (Sortase A), SUB0145 (Lactoferrin binding protein), SUB1095 (Collagen like surface-achored protein), SUB1635 (SUAM protein) i SUB1785 (PauA Streptokinase precursor). Però estudis posteriors, van provar que aquests gens es trobaven conservats tan en aïllats de casos clínics com de casos no clínics, de manera que la seva presència no permetia diferenciar aquelles soques virulentes de les no virulentes (Hossain et al., 2015), suggerint que altres factors com les co-infeccions, la genètica i l'estat immunològic de l'hoste en el moment de la infecció podien jugar un paper important en l'establiment d'una IMI.

1.3.3.1. Biofilm com a factor de virulència

Un biofilm és una comunitat de microorganismes capaç de créixer adherida sobre una superfície gràcies a la producció d'una matriu extracel·lular que embolcalla i manté unides les cèl·lules. La formació d'aquesta comunitat sèssil s'ha descrit com un important

mecanisme de virulència i supervivència bacteriana en moltes espècies, ja que confereix tolerància a les defenses de l'hoste i al mateix temps als tractaments antibiòtics convencionals, donant lloc a infeccions cròniques (Costerton et al., 1999) (Figura 8).

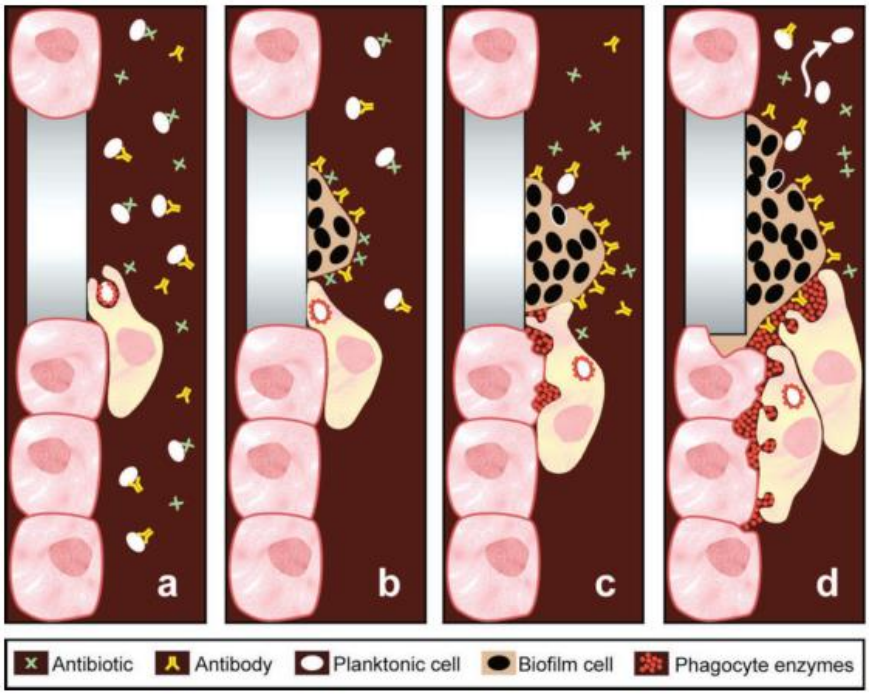

Figura 8. Esquema del mecanisme pel qual el biofilm confereix resistència a les defenses de l'hoste i als antibiòtics. (A) Els bacteris planctònics poden ser opsonitzats i fagocitats per les cèl·lules fagocítiques del sistema immunitari i són susceptibles a l'efecte dels agents antimicrobians. (B) Els bacteris troben l'oportunitat d'adherir-se a una superfície i créixer formant biofilm, establint una comunitat on els anticossos i els agents antimicrobians no hi tenen accés. (C) Les cèl·lules no poden fagocitar els bacteris que s'han establert en comunitats. (D) Els enzims alliberats per les cèl·lules fagocítiques danyen el biofilm i els bacteris planctònics que s'alliberen es poden disseminar i infectar teixits propers. Adaptació de *"Diagram of a medical biofilm"* de Costerton et al.1999.

La capacitat de formació de biofilm és doncs un important mecanisme de virulència en diferents patologies de l'àmbit veterinari (Jacques, Aragon, i Tremblay, 2010). Específicament en la glàndula

mamària bovina, els bacteris protegits per aquesta matriu extracel·lular esdevindrien resistents a l'activitat fagocítica de les cèl·lules de l'hoste i als agents antimicrobians. Aquesta resistència, els hi suposaria un avantatge a l'hora d'adherir-se i colonitzar l'epiteli mamari donant lloc així a infeccions persistents (Melchior, Vaarkamp, i Fink-Gremmels, 2006).

En aquesta mateixa línia, diversos autors han demostrat la importància del biofilm com a factor de virulència en el desenvolupament d'una IMI. Per exemple, Fox i els seus col·laboradors (2005) van observar com les soques de *S. aureus* aïllades de la glàndula mamària, tenien una major habilitat de formar biofilm *in vitro* que aquelles aïllades de la pell de l'animal o de la línia de munyida. Posteriorment, un altre equip d'investigadors va observar com els aïllats de casos clínics de mamitis bovina causada per *S. uberis,* exhibien una habilitat superior de formar biofilm *in vitro* que els aïllats de vaques sanes (Crowley, Leigh, Ward, Lappin-Scott, i Bowler, 2011). Al 2014, Elhadidy i Zahran, van observar com la formació de biofilm tenia un paper important en l'adhesió i invasió de *Enterococcus faecalis* a l'epiteli mamari boví també *in vitro*. Finalment, tant Gogoi-Tiwari i col·laboradors al 2015, com Schönborn and Krömker al 2016, van aconseguir posar de manifest la rellevància del biofilm de *S. aureus* com a factor de virulència en els seus estudis *in vivo*. Els primers, van demostrar que una vacuna basada en el biofilm de *S. aureus* exhibia major eficàcia que una vacuna basada en cèl·lules crescudes de forma planctònica en un model murí. Mentre que els segons, van aconseguir detectar en el teixit epitelial de la glàndula mamària de vaques naturalment

infectades, poly-N-acetyl-b-(1,6)-glucosamine (PNAG), un polisacàrid que forma part del biofilm de *S. aureus*, demostrant així que durant algun moment de la infecció s'havia desencadenat la formació de biofilm.

Malgrat aquestes observacions, altres autors han descrit que en els seus estudis no hi ha evidències que la formació de biofilm *in vitro* estigui relacionada amb la virulència de *S. uberis in vivo* (Salomäki et al., 2015; Tassi, McNeilly, Sipka, i Zadoks, 2015). Encara que una explicació possible per aquesta falta de correlació seria que la formació de biofilm *in vitro*, és altament dependent del medi de cultiu (Varhimo et al., 2011). De manera que la manca de formació de biofilm *in vitro* d'una soca, no té perquè significar que *in vivo* no en pugui produir, ja que com alguns autors han demostrat, l'addició de caseïna o llet al medi de cultiu, resulta en la formació de biofilm de totes les soques de *S. uberis* analitzades (Crowley et al., 2011; Varhimo et al., 2011).

Així doncs, davant de les evidències sobre la importància del biofilm pel desenvolupament d'una IMI, en aquest treball es va decidir centrar la cerca de candidats vacunals en els components tant proteics com no proteics del biofilm de *S. uberis*. La descripció i resultats d'ambdues línies de treball, es descriuen en el capítol 2 i 3 de la present tesi doctoral.

1.4 Desenvolupament de vacunes

En l'actualitat, el desenvolupament de vacunes és un repte molt important per a la indústria farmacèutica. La creixent aparició de

resistències a antibiòtics i l'escassetat d'alternatives als tractaments convencionals, fa pensar en la vacunació com a l'eina preventiva per excel·lència de cara al futur.

El desenvolupament d'una vacuna però és molt complex, passant des d'etapes inicials de recerca bàsica i escalat industrial, a estudis preclínics i clínics abans de l'obtenció d'una llicència comercial. Depenent del tipus de vacuna, la durada del desenvolupament d'una vacuna veterinària pot ser d'entre 5-10 anys abans de la seva comercialització. A continuació en la taula 6 es mostra un resum de les principals etapes de desenvolupament d'una vacuna.

Taula 6. Etapes principals de desenvolupament d'una vacuna.

Fase	Descripció
Fase 1	Recerca preliminar. Selecció de l'antigen i l'adjuvant, desenvolupament de tècniques analítiques de control de qualitat i escalat industrial de la formulació.
Fase 2	Estudis preclínics d'eficàcia i seguretat en l'espècie de destí en condicions controlades.
Fase 3	Estudis clínics d'eficàcia i seguretat en l'espècie de destí en condicions reals de camp.
Fase 4	Elaboració del dossier de registre. Avaluació i aprovació de la vacuna per part de l'autoritat competent.

Durant la fase 1, és important comprendre els mecanismes de patogenicitat del microorganisme per seleccionar l'antigen adequat per conferir protecció en front a la malaltia. Per altra banda, cal seleccionar també l'adjuvant adequat per estimular el tipus de

resposta immune necessària i finalment, un cop seleccionada la formulació, cal fer les proves necessàries per poder produir la fórmula desenvolupada a nivell industrial. A continuació en la fase 2, s'inicien els estudis preclínics, que consisteixen en proves d'eficàcia i seguretat en l'espècie de destí en condicions experimentals. Aquestes proves serviran per determinar si la vacuna és eficaç i segura en condicions controlades. Un cop superada aquesta etapa, s'entra la fase 3 o fase d'assajos clínics, on la vacuna és testada en l'espècie de destí en condicions reals de camp amb els permisos de les autoritats sanitàries pertinents i d'acord amb la regulació de cada país. Finalment, un cop superada la fase 3, la vacuna entra en fase 4 o fase de registre, on es recopila tota la informació generada durant el desenvolupament del producte juntament amb els resultats obtinguts en les fases 2 i 3, per elaborar un dossier que es presentarà a les autoritats competents per a la seva avaluació. A nivell europeu, l'Agencia Europea del Medicament (EMA) és l'encarregada de coordinar l'avaluació i l'aprovació de vacunes per tota la UE juntament amb les agències del medicament de cada país.

1.4.1. Desenvolupament d'una vacuna contra la mamitis bovina causa per *Streptococcus uberis*

Durant molts anys, diferents grups de recerca han treballat en el desenvolupament d'una vacuna contra la mamitis bovina causada per *S. uberis*, però cap estratègia ha resultat eficaç fins a dia d'avui. Finch i col·laboradors (1994) van aconseguir desenvolupar una formulació que conferia protecció davant una infecció experimental intramamària amb una soca homòloga. Posteriorment, el mateix grup va treballar en una vacuna que combinava l'administració subcutània

i intramamària, i malgrat demostrar eficàcia també contra una soca homòloga, no va aconseguir bons resultats en una IMI amb una soca heteròloga (Finch, Winter, Walton, i Leigh, 1997).

Posteriorment, Leigh i els seus col·laboradors (1999), van aconseguir desenvolupar una vacuna de subunitat basada en el PauA, amb la que van aconseguir un 37.5–62.5% de protecció en front a la mamitis clínica causada per *S. uberis*, però no existeix cap publicació posterior amb resultats d'eficàcia en una prova clínica.

Finalment, treballs més recents amb la proteïna SUAM, demostren que la vacunació amb aquesta proteïna és capaç d'induir la formació d'anticossos específics que redueixen l'adhesió i la internalització de *S. uberis* a les cèl·lules MEC (Prado et al., 2011). Posteriorment, el mateix grup de recerca va demostrar que la infusió intramamària de *S. uberis* opsonitzat amb anticossos anti-SUAM, redueix els signes clínics de mamitis i el recompte bacteriològic en comparació amb l'administració de *S. uberis* no opsonitzat en vaques en lactació (Almeida et al., 2015). Tanmateix, malgrat els bons resultats d'aquests treballs, no es disposen d'estudis que demostrin l'eficàcia de SUAM com a candidat vacunal.

Podem concloure doncs que, fins al moment, cap estratègia ha resultat en una vacuna eficaç i segura per a la prevenció de la mamitis bovina causada per *S. uberis*, i que en l'actualitat, no es disposa de cap vacuna comercial amb eficàcia clínica provada. Així doncs, les mesures de control actual d'aquesta patologia es limiten a la implementació del *five-point control plan*, que per altra banda ha

demostrat tenir una eficàcia molt limitada en el control de la prevalença i incidència de mamitis per *S. uberis* (Leigh, 1999).

Per tant, el desenvolupament d'una vacuna com aquesta, és un gran repte per la indústria farmacèutica veterinària a nivell mundial. Concretament en aquesta tesi doctoral, es presenten una sèrie de treballs que s'emmarquen en les fases 1 i 2 del desenvolupament d'una vacuna contra la mamitis bovina causada per *S. uberis*

Objectius de la tesi doctoral

2. Objectius de la tesi doctoral

Els objectius del treball realitzat en aquesta tesi doctoral s'estructuren en 3 capítols que s'emmarquen en les fases 1 i 2 del procés de desenvolupament d'una nova vacuna contra la mamitis bovina causada per *Streptococcus uberis:*

- Capítol 1. Desenvolupament d'un model d'infecció experimental intramamària capaç de reproduir la mamitis bovina causada per *S. uberis* en vaques en lactació. Aquesta fita es requereix per realitzar proves d'eficàcia de vacunes experimentals formulades amb els candidats vacunals resultants dels estudis descrits en els capítols 1 i 2.

- Capítol 2. Anàlisi proteòmic diferencial per detectar antígens proteics associats a la formació de biofilm de *S. uberis* i anàlisi de la seva eficàcia en un model murí.

- Capítol 3. Obtenció i caracterització d'un antigen no proteic associat a la formació de biofilm de *S. uberis* i anàlisi de la seva eficàcia en una prova preclínica en vaques en lactació.

Resultats

3. Resultats

Capítol 1. Different infection kinetics after an intramammary challenge with *in vitro Streptococcus uberis* biofilm forming and non-biofilm forming strains in dairy cattle

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Abstract

The aim of this study was to clarify whether the *in vitro* biofilm formation ability of *S. uberis* has any impact on the development of an intramammary infection (IMI). With this objective in mind, two *S. uberis* strains isolated from clinical mastitis cases were selected, one as a biofilm forming strain (SU2H) and the other as a non-biofilm forming strain (0140J) on a polystyrene 96-well microplate assay. Then, two groups of four cows each received an intramammary challenge with the same dose of each strain. Following the intramammary challenge, clinical signs of mastitis, milk production, somatic cell count (SCC) rectal temperature and bacterial cell count in milk were recorded daily, for sixteen days post-infection. Results showed that the two strains exhibited clear differences in pathogenicity. Significant differences in bacterial count, SCC, temperature and milk yield between groups were recorded, suggesting that biofilm formation ability could confer a different colonisation and persistence strategy in the mammary gland, although it cannot be ruled out that other virulence factors could be involved.

Keywords: *Streptococcus uberis*, mastitis, biofilm, dairy cow

Introduction

Streptococcus uberis is one of the major causative pathogens of clinical and subclinical mastitis in dairy cattle. It has been considered one of the main causes of clinical mastitis in countries such as the United Kingdom, Canada, the USA and the Netherlands, and the main cause of clinical mastitis in New Zealand and Australia,

sometimes reaching 63% of all clinical cases of mastitis (Hillerton, Shearn, Teverson, Langridge, i Booth, 1993; Hogan i Smith, 1997; McDougall et al., 2007; S. M. Wang et al., 1999)

Many studies have been performed in order to discover the main virulence factors of *S. uberis,* and some candidates have been proposed, although their relevance in the development of bovine mastitis is still unclear: antiphagocytic factors which include capsule, neutrophil toxin, M-like protein and R-like protein; potential virulence factors released extracellularly such as hyaluronic acid capsule, hyaluronidase, uberis factor (Oliver, Almeida, i Calvinho, 1998) and several adhesins such as *S. uberis* adhesion molecule (SUAM) which is involved in the internalisation of mammary epithelial cells through lactoferrin (Lf) binding (Almeida *et al.* 2006).

In the past few years, several publications have described biofilm formation as an important mechanism for the establishment of bovine mastitis. For instance, Elhadidy M *et al.* (2014) described biofilm as playing an important role in the adhesion and invasion of *Enterococcus faecalis* in bovine mammary epithelial cells. Furthermore, Costerton *et al.* (1999) stated that biofilms are involved in the reduction of bacterial susceptibility to phagocytosis and to antibiotic therapies, which ultimately leads to the establishment of persistent intramammary infections (IMI) (Melchior *et al.* 2006). Supporting these data, Fox *et al.* (2005) reported that in the case of *Staphylococcus aureus,* strains isolated from the mammary gland were more capable of forming biofilm *in vitro* than strains isolated

from extra-mammary sources such as teat skin and milking unit liners.

In relation to *S. uberis*, several authors have demonstrated that its *in vitro* biofilm formation ability is strongly dependent on culture conditions (Varhimo *et al.* 2011; Tassi *et al.* 2015; Schönborn *et al.* 2017). Furthermore, Crowley *et al.* (2011) demonstrated that *S. uberis* isolated from clinical cases of bovine mastitis produced significantly more biofilm than an isolate obtained from a healthy bovine mammary gland. However, in a different direction, Tassi and colleagues (Tassi *et al.* 2015) reported that, although resistance to macrophage killing and the ability to adhere to mammary epithelial cells were *in vitro* traits of *S. uberis* that correlate with its virulence *in vivo*, no relationship between bacterial virulence *in vivo* and biofilm formation *in vitro* was found in their studies.

Taking into account the discrepancies between the results of Crowley *et al.* (2011) and Tassi *et al.* (2015) regarding the relevance of biofilm formation *in vitro* and the virulence of *S. uberis* strains, the objective of this study was to compare the pathogenicity of two *S. uberis* isolates from clinical mastitis with different biofilm formation ability *in vitro*, through an experimental IMI set up in dairy cows. With this purpose in mind, bacterial count, somatic cell count (SCC), clinical signs of mastitis, rectal temperature and milk production were analysed throughout the entire study after challenge.

Materials and methods

Challenge strains and culture growth conditions

The 0140J strain (ATCC-BAA-854) and SU2H strain (Hipra collection), were both isolated from clinical cases of bovine mastitis in the UK and selected in further biofilm formation experiments in order to represent a non-biofilm forming strain (0140J) and a biofilm forming strain (SU2H). To study the bacterial growth dynamics *in vitro*, *S. uberis* strains were cultured in Tryptic Soy Broth (Becton Dickinson France, S.A) containing 0.5% Yeast Extract (Becton Dickinson France, S.A) (TSB+YE) overnight at 37ºC (18-20 h). TSB+YE cultures were diluted 1:100 in fresh medium and the cell growth at 37ºC was analysed by determining the colony forming units (CFU) in Columbia III Agar with 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany).

Biofilm assays

The bacterial inoculum for the biofilm assay was prepared as follows: lyophilised cultures in glass vials were thawed, suspended with 1 ml of ultrapure sterile water, plated onto Columbia agar and incubated overnight at 37ºC; after confirmation of viability and purity, 3 single colonies were inoculated in 20 ml of TSB+YE and incubated at 37ºC for 16 h. The ability of *S. uberis* to form biofilm was evaluated using 96-well polystyrene plates (Thermo Fisher Scientific) as previously described by Sandberg et al. (2008) with the following modifications: 16 h cultures of 0140J and 6121 were diluted 1:100 in TSB+YE and 200 µl of the suspensions were used to inoculate 96-well polystyrene plates; fresh TSB+YE media without bacterial inoculum was

inoculated as a negative control (C-); cells were grown for 24 h at 37ºC; then, planktonic suspensions were removed and the wells were washed three times with phosphate buffered saline (PBS) and dried for 1 hour at 37ºC; the cells were heat-fixed and stained with a 0.5% (v/v) Crystal violet solution (Merck) for 10 minutes at room temperature; the stain was removed and the wells were washed 4 times with 300 µl PBS; stained biofilms were diluted in 200 µl 96% ethanol and then incubated under orbital agitation for 2 h; finally, the optical density at 595 nm was measured using a microplate reader (TECAN FRANCE, S.A). The experiment was conducted on three days using 8 wells in each plate for the culture of one strain. The optical density values for 24 wells were obtained for each isolate and used to determine the mean value. When the mean value of the optical density values for 24 wells was statistically higer than the mean of the optical density values for 24 wells of the C-, the strain was considered a biofilm producer strain.

Animals

The experimental IMI study was performed at Laboratorios Hipra S.A facilities (Amer, Girona) and it was approved by the Ethical Committee for Animal Experimentation of Laboratorios Hipra S.A in compliance with Directive 2010/63/EU on the protection of animals used for scientific purposes (adopted on 22 September 2010). A total of 8 clinically healthy Holstein primiparous cows were selected for the study. All cows were in the first early lactation (30-60 days in milk on the day of challenge). The somatic cell count (SCC) for each quarter was measured at the time of selection and

gave a result of <150,000 cells/ml at the time of purchase. Five (D-5), two (D-2) and one (D-1) day before the challenge, quarter milk samples from all animals were negative for pure bacterial culture and SCC values remained under 150,000 cells/ml. All the animals were housed together in a single pen, with straw bedding and they were fed with a total mixed ration for cows in early lactation. Water was offered *ad libitum* throughout the entire study. The cows were milked twice a day, at 08:00 h and 16:00 h using a dedicated milking unit per group. After each milking, all teats and teat cups were dipped in iodine-based teat disinfectant in order to avoid cross-contamination between cows.

Challenge Study Design

The study was performed following a randomised double-blind design, comparing an experimental IMI with 0140J and SU2H strains. The two groups were identified as group A (0140J) and group B (SU2H) during the study. The animals were included in one of the two groups via randomised distribution based on milk production as follows: before the challenge, the animals were ranked as a function of their milk production in order to assign paired animals to four ranks as a function of milk yield. A random number was then assigned to each animal; those animals with the lowest random number in each rank were assigned to group A, whereas the rest were assigned to group B. The staff involved in the clinical evaluation and the laboratory technicians were not aware of the treatment received by each individual animal or the treatment coding. All samples were labelled including the corresponding animal's ear tag number. The

outcomes considered in the present study were: bacterial count, clinical signs of mastitis, rectal temperature, SCC and daily milk production after challenge.

Intramammary Challenge

Lyophilised cultures in glass vials were thawed, suspended with 1 ml of ultrapure sterile water plated on Columbia III Agar with 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany) and incubated overnight at 37ºC. After confirmation of viability and purity, single colonies were seeded in a TSA+YE media and incubated at 37ºC for 24 h. Bacterial growth was suspended in chilled PBS and harvested by centrifugation for 20 min at 5,000 x g at 4ºC. The bacterial suspensions were then adjusted to 1 x 10^2 CFU/ml. The results obtained from the bacterial count for each strain were 1.56 x 10^2 CFU/ml for 0140J and 1.24 x 10^2 CFU/ml for SU2H. Each animal was challenged by the intramammary infusion of 5 ml of inoculum (0140J or SU2H) in the rear right quarter (RR), after morning milking as follows: the tip of the teat to be infected was scrubbed vigorously for 10-15 seconds with a paper towel soaked with alcohol 70%; a 10 ml syringe was filled with 7 ml of inoculum and connected to a sterile 15 cm cannula; this cannula was filled with the inoculum suspension before being inserted into the mammary gland, and then 5 ml of inoculum suspension was infused into the infected quarter; a slight massage was performed by palpation of the infected quarter for 30 sec; the teat tip was cleaned again with an alcohol towel; finally, iodine solution was applied, soaking the infected teat to avoid any other possible infection.

Milk sample collection, bacterial count and SCC

Milk samples from all infected and non-infected quarters were collected 5, 2 and 1 day before the challenge (D-5 am, D-2 am and D-1 am), twice a day from the day of the challenge to 5 days after (D0 am to D5 pm) and during the morning milking from 6 to 16 days after the challenge (D6 am to D16 am) as follows: the end of the teat was vigorously scrubbed for 10-15 seconds with a paper towel soaked with 70% (vol/vol) ethanol, a few streams of milk were then discarded; the cap from the sampling tube was removed and maintained facing downward, while the sampling tube was positioned at approximately a 45 degree angle. Two to four streams of milk were aseptically collected into the pre-labelled sterile plastic tubes, avoiding contact between the tube and the teat. These samples were kept on ice (in the milking parlour) during sampling, after that, they were stored at -20ºC and later cultured for mastitis pathogens. Quantitative bacteriological analysis of the milk samples from infected and non-infected quarters was performed by spreading 100 µl of homogenised milk onto Columbia III Agar with 5% sheep blood plates by means of an Eddy Jet 2 Spiral Plater (IUL Instruments, Spain). After 24±4 h of incubation at 37±2˚C, the identity of the *S. uberis* strain was confirmed by analysing colony morphology (size, colour), Gram stain and oxidase and catalase reactivity. Colony forming units per ml (CFU/ml) of each sample, compatible with *S. uberis*, were estimated by using a Flash and Go colony counter reader and software (IUL Instruments, Spain). Negative samples (with no bacterial isolation) were confirmed by bacteriological culture of 250

µl of homogenised milk samples onto Columbia III Agar with 5% sheep blood plates.

A second sample, for SCC analysis, was collected from all quarters following the same procedure described above, but using non-sterile vials containing a broad spectrum (bronopol and Natamycin combination) preservative tablet (Broad spectrum microtabs II; Advanced Instruments Inc., MA, USA). These samples were also kept on ice (in the milking parlour) during sampling, after that they were stored at 4-6ºC and shipped to a commercial laboratory (Associació Interprofesional Lletera de Catalunya, ALLIC, Cabrils, Spain) twice weekly for SCC analysis by Fossomatic™ FC flow cytometry (Foss Iberia, S.A, Spain). These results were reported electronically via email.

Clinical assessments

All the animals were closely observed on D-5, D-2, D-1 and daily from D0 until D16 to assess visual abnormalities in the milk and in the mammary gland in all quarters and to record rectal temperature and milk production for each animal.

Visual abnormalities in the milk were assessed before milking according to the following scoring: (0) normal colour and normal density, white without abnormalities; (1) slightly affected, where small clots could be observed (<5 mm in diameter) and/or slight abnormal colour (yellowish milk); (2) moderately affected, where clots or aggregates were larger (>5 mm in diameter) and/or the density of milk increases; (3) severely affected, where blood was present in milk samples. The effect on the mammary gland was

assessed visually and by palpation after milking according to the following scoring: (0) Normal mammary gland, without signs of oedema, sclerosis, swelling, abscesses or gangrene; (1) Mildly affected, less than 25% of the challenged quarter of the mammary gland affected with swelling, oedema or sclerosis; (2) Moderately affected, between 25% and 75% of the challenged quarter of the mammary gland affected with swelling, oedema or sclerosis; (3) Severely affected, more than 75% of the challenged quarter of the mammary gland affected with swelling, oedema or sclerosis and/or with some signs of gangrene. Furthermore, a composite score of the total clinical signs of mastitis was derived from the maximum score for either the visual abnormalities in the milk or for the effects on the mammary gland on each day of the post challenge evaluation. If the score for the mammary gland and visual abnormalities in milk were the same, this value was used.

Animals were milked twice a day using a dedicated milking unit per group in order to avoid cross-contamination between cows. Milking units were attached just after udder preparation and were manually removed after milking; afterwards all teats were dipped in iodine-based teat disinfectant. Milk weights were recorded from the in-line weighing jar and the milking units were manually black-flushed with a dilute chlorhexidine digluconate solution (OXA MAST P, Cygyc Industria Quimica, S.A., Spain). Milk production was registered from D-5 until the end of the study for each animal.

Statistical Analysis

Data on biofilm formation were compared using the MIXED procedure *t*-test from SAS (SAS Institute, 2008). The data are shown as mean ± standard error of the mean (SEM). The slope of the line (growth rate) whose equation was determined by linear regression as representing the exponential growth phase was used to calculate the generation time (G) as follows: slope = 0.301 / G. Statistical analysis was performed taking into account only those quarters (for bacterial count, SCC and clinical signs of mastitis) or animals (for temperature and milk production) that developed clinical mastitis, 3 out of the 4 infected with the 0140J strain and the 4 infected with the SU2H strain. To satisfy assumptions of normality, bacteriological count and SCC data from infected quarters were log-transformed. To evaluate the effect of the challenge strain on bacterial count, SCC, temperature and milk production, the data were analysed using the PROC MIXED procedure *t*-test from SAS (SAS Institute, 2008). The model included the fixed effects of strain, sampling time, and strain-by-sampling time interaction, and the random effect of animal within strain. Compound symmetry was selected as the variance-covariance matrix structure for the bacterial count, SCC and rectal temperatures data on the basis of best fit according to Schwarz's Bayesian information criterion. The clinical signs of mastitis score per quarter was the maximum score for either visual abnormalities in milk or for the effects on the mammary gland. The comparison between the vaccinated and mock-vaccinated group was conducted in an all time-point assessment using the Wilcoxon rank sum test within PROC NPAR1WAY from SAS (SAS Institute, 2008) as these data were not parametric. The

area under the curve (AUC) for the clinical signs of mastitis data was calculated obtaining the mean value per animal from D0 to D16. Then, the AUC data for the vaccinated group were compared to the mock-vaccinated group using the MIXED procedure *t*-test from SAS (SAS Institute, 2008). All values reported are least squares means. Significance was declared at $P \leq 0.05$, and trends reported if $0.05 < P \leq 0.10$.

Results

Biofilm assays

The biofilm formation assay performed in a 96-well microtiter plate showed that the SU2H strain exhibits significantly greater biofilm formation ($P < 0.05$) than the 0140J strain and the C-, which consisted of non-inoculated culture media. Furthermore, 0104J showed no differences from the negative control which indicates that no biofilm was produced in the *in vitro* test (Figure 1).

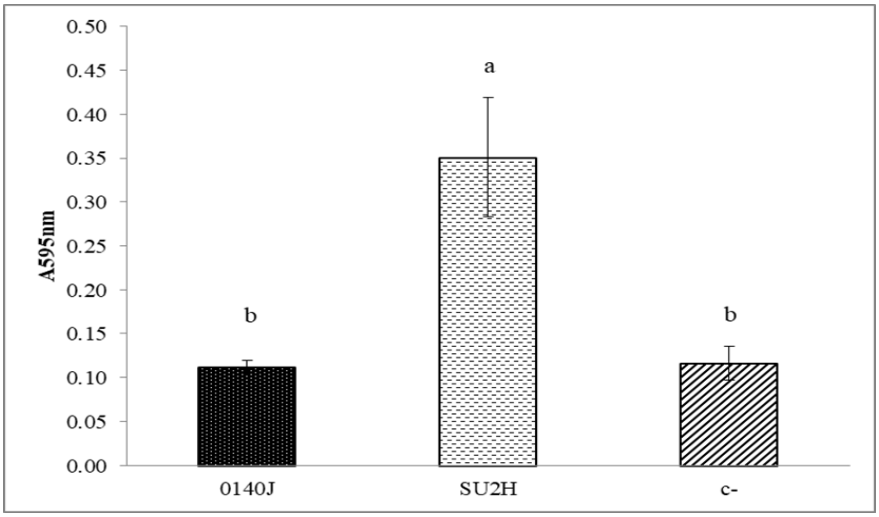


Figure 1. Biofilm formation of *Streptococcus uberis* strains 0140J and SU2H in microtiter plates with TSB+YE. The media was included as a negative control (c-). The values represent the mean OD after 24 h of culture at 37ºC and the standard deviation of the mean. The assay was conducted on 3 different cultures of each strain and for each assay the result was based on the average of 8 wells. Different superscripts (a, b) indicate significant differences ($P < 0.05$).

Bacterial growth in vitro and after the experimental intramammary infection

After the challenge, both groups displayed *S. uberis* positive cultures in infected quarters as detailed below. In contrast, non-challenged quarters were never bacteriologically positive for *S. uberis* after the challenge up to the end of the study (data not shown). After IMI, the SU2H strain was isolated in all challenged quarters (100% incidence). The SU2H strain exhibited an exponential bacterial growth phase until 3 days after infection, when the peak number of colony-forming units (CFU/ml) was reached (2.84×10^5 CFU/ml) (Figure 2). On the other hand, after challenge with the 0140J strain, it was isolated in 3 out of 4 quarters (75% incidence) and then, a

longer exponential phase from the first milking after infusion at 0+8h until 5 days after was described. The highest CFU/ml value was reached on Day 9 after the challenge (1.39 x 10^8 CFU/ml) (Figure 2). The bacterial count for the quarters infected with 0140J was significantly greater ($P < 0.05$) than that for the quarters infected with SU2H on Day 1, Day 4 and from Day 5 until the end of the study. After the maximum mean bacterial count value, shedding was maintained throughout the study between 1.8 x 10^2 – 6.53 x 10^4 CFU/ml for the SU2H strain and 1.14 x 10^7 – 9.81 x 10^7 CFU/ml for the 0140J strain. Furthermore, considering the overall result, quarters infected with the 0140J strain showed a significantly ($P < 0.05$) greater average for the bacteriological count from Day 0 to Day 16 compared to quarters infected with the SU2H strain (5.25 ± 0.32 vs 2.96 ± 0.28 Log CFU/ml, respectively).

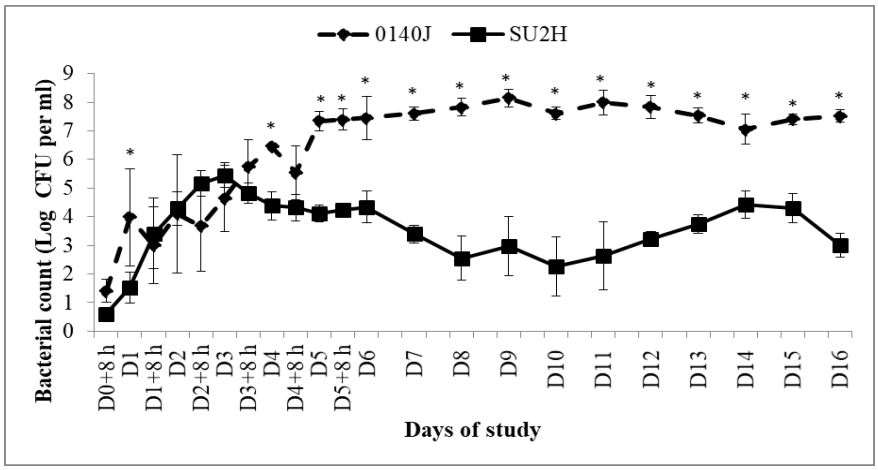
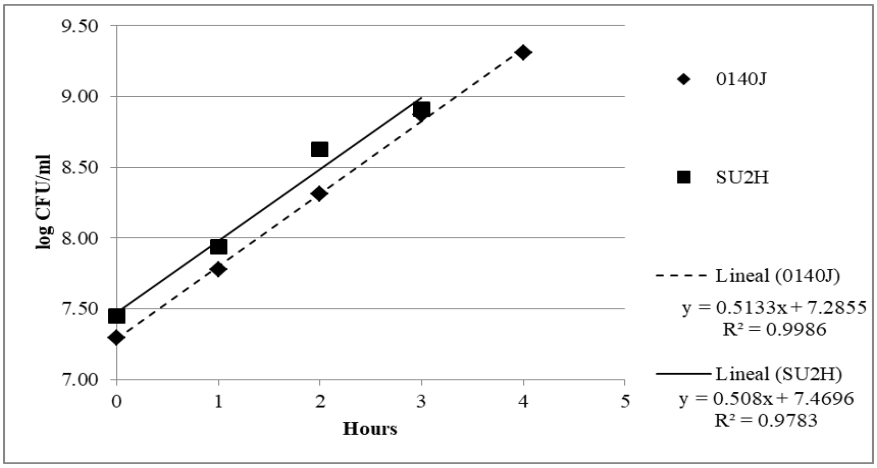


Figure 2. Mean and standard error of the mean (SEM) of bacterial counts in milk throughout the study following intramammary infection (IMI) with the 0140J (♦) and SU2H (■) strains. Calculations have been performed with data for quarters excreting *Streptococcus uberis*. *Superscript indicates significant differences ($P < 0.05$).

Furthermore, the *in vitro* and *in vivo* generation times (G) were calculated for both strains during the exponential growth phase. The *in vitro* G calculations were based on laboratory cultures in TSB+YE media (Figure 3A). The *in vivo* generation time calculations were based on the number of viable bacteria recovered from milk from infected quarters at each sampling time during the bacterial exponential phase, as a combined effect of bacterial replication and clearance (mainly due to the host defences and the daily milking) (Figure 3B).

A)



B)

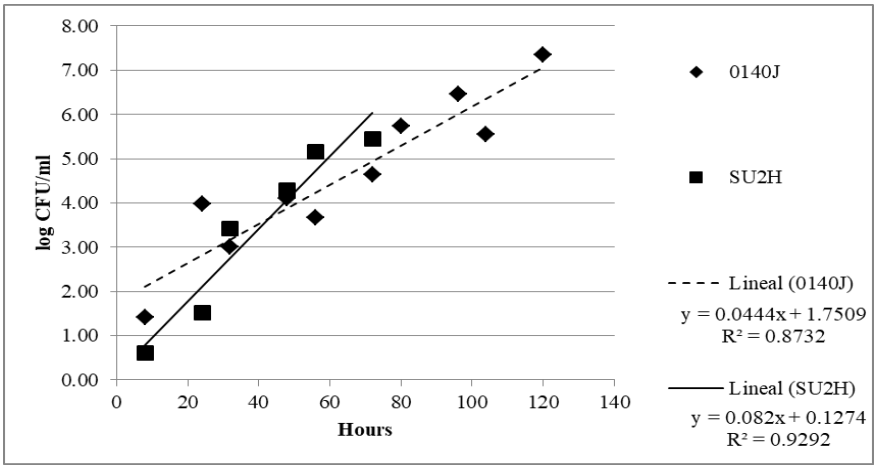


Figure 3. (A) *In vitro* exponential growth phase of both 0140J (♦) and SU2H (■) strains in TSB culture media. (B) Mean of *in vivo* exponential growth phase of 0140J (♦) and SU2H (■) strains in milk from infected quarters.

The results showed that the *in vitro* generation time of both strains was identical and lower than the *in vivo* growth. Moreover, a notable difference was observed in the *in vivo* generation time between both strains (Table 1).

Table 1. Mean generation time *in vitro* and *in vivo* (hours)

Strain	*in vitro* (TSB+YE)	*in vivo*
0140J	0.59	6.78
SU2H	0.59	3.67

Somatic cell count (SCC)

The SCC in the non-infected quarters remained < 200,000 cells/ml throughout the entire study. For the infected quarters, the group challenged with the SU2H strain showed its maximum increase on Day 3 (a.m. and p.m.), coinciding in time with the maximum increase in bacteriological count, which then remained stable until the end of the study. On the other hand, the mean SCC for the 0140J challenged group exhibited an increase from Day 0 until Day 5, and from Day 5 onwards it maintained uniform values until Day 15, when it started to decrease. Quarters infected with SU2H described significantly ($P < 0.05$) greater SCC compared to those infected by the 0140J strain on Days 3, 3+8h and 16 after challenge. However, considering the overall results, quarters infected with the 0140J strain described significantly ($P < 0.05$) greater SCC from Day 0 to Day 16 compared to quarters infected with SU2H (6.52 ± 0.15 vs 6.24 ± 0.11 LogSCC/ml, respectively). Hence, despite the significant differences displayed between groups in bacteriological count and SCC, the SCC remained >200,000 cells/ml in all infected quarters from which bacteria were recovered in both groups throughout the study, suggesting that the infection persisted in both groups until the end of the observation period (Figure 4).

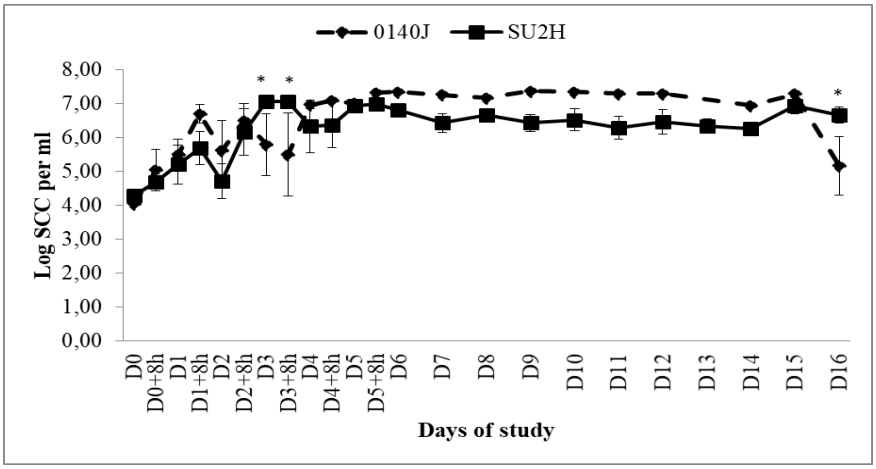


Figure 4. Mean and SEM of somatic cell count in response to an intramammary challenge with 0140J (♦) and SU2H (■) strains throughout the study. Calculations have been performed with data for quarters excreting *Streptococcus uberis*. *Superscript indicates statistically significant differences ($P < 0.05$). SSC from 0140J was not determined on Day 13.

Clinical signs of mastitis

Non-challenged quarters did not show any clinical signs of mastitis during the study (data not shown). Figure 5 shows the average of these scores per group and for every sampling of the study, from D-5 to D16. As observed for the bacterial count and SCC, 5 days after the challenge, both groups had a similar increase in clinical signs. However, after that, the quarters challenged with the SU2H strain displayed lower values than the 0140J infected group. No significant differences were observed between the two groups during the assessment time points. However, the quarters infected with the 0140J strain described numerically greater clinical scores compared to the quarters infected with SU2H from Day 6 until the end of the study (Figure 5). Consequently, the overall result for the quarters

infected with the 0140J strain was also numerically greater ($P = 0.13$) compared to the quarters infected with SU2H (2.13 ± 0.19 and 1.42 ± 0.31 score, respectively).

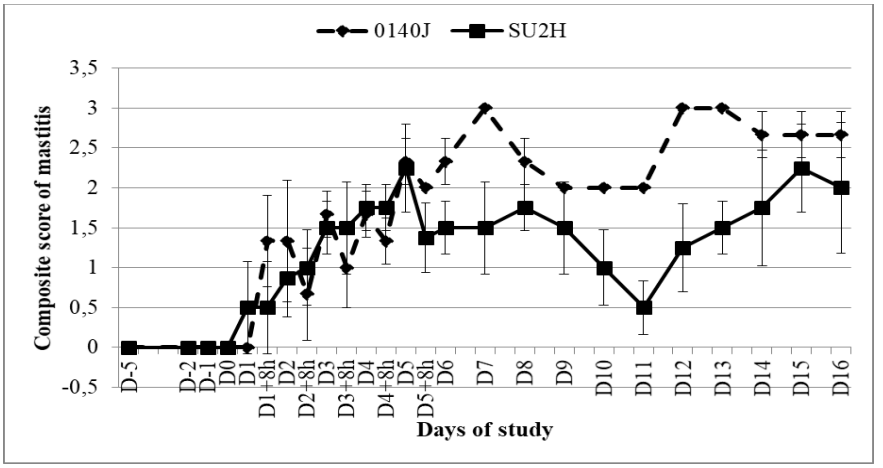


Figure 5. Mean and SEM of the composite score for clinical signs for mastitis throughout the study in response to an intramammary challenge with 0140J (♦) and SU2H (■) strains (Day 0). Calculations have been performed with data from quarters excreting *Streptococcus uberis*.

Temperature

Rectal temperatures of both groups were similar before the challenge, but some differences were recorded during the observation period after the experimental infection. The SU2H challenged group displayed its maximum temperature increase at D3+8 h after challenge, when this group described significantly ($P < 0.05$) greater rectal temperatures than the 0140J challenged group. This fact correlates with the fact that 8 h before, the same group displayed a peak in bacteriological count. After that increase, the rectal temperature of the SU2H group decreased until D6. After D6 the mean rectal temperatures of the SU2H group remained below the

mean for the 0140J group until the end of the study. Furthermore, animals infected with 0140J described several peaks during the study, where their rectal temperature was significantly greater ($P < 0.05$) compared to the SU2H challenged group on Day 1+8h, 8, 11 and 12 after challenge (Figure 6). However, no significant differences were observed between the two groups in relation to the overall results.

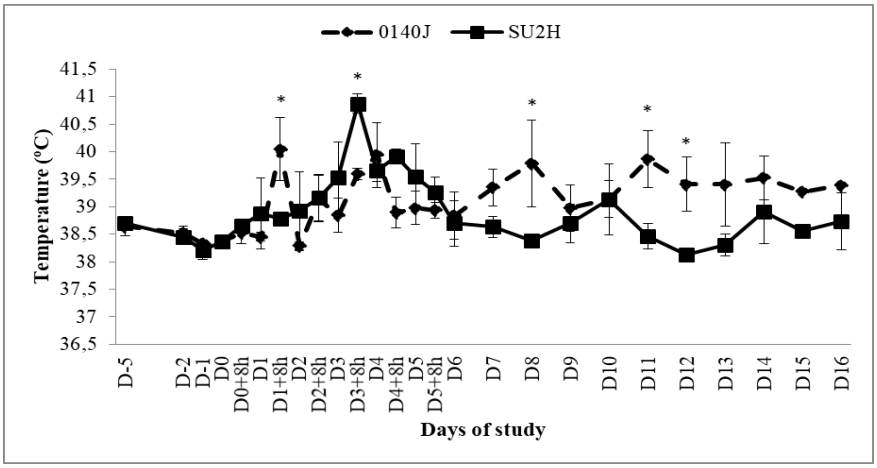


Figure 6. Mean and SEM of rectal temperatures in response to an intramammary challenge with 0140J (♦) and SU2H (■) strains throughout the study. Calculations have been performed with data from quarters excreting *Streptococcus uberis*. *Superscript indicates significant differences (P <0.05).

Milk production

Results for milk production at the individual animal level indicated that both groups suffered a similar decrease from Day 0 to Day 5. But then, the animals challenged with the SU2H strain showed an increase from Day 6 to Day 16. On the other hand, this reduction in milk production was noticed in the 0140J challenged group until Day 12 (Figure 7). Consequently, animals challenged with SU2H showed significantly ($P < 0.05$) greater milk production compared to the

0140J challenged group on Days 8, 10, 12, 13, 14 and 15 after the challenge. No significant differences were noticed in the analysis of overall milk production data. However, by the end of the study, the milk production levels for all the animals had recovered to similar levels as those observed before the challenge.

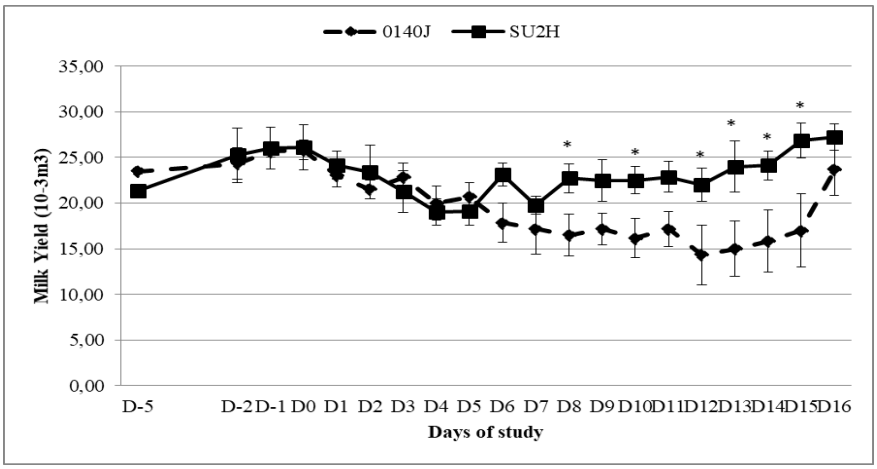


Figure 7. Mean and SEM of the milk yield in response to an intramammary challenge with 0140J (♦) and SU2H (■) strains throughout the study. Calculations have been performed withdata from quarters excreting *Streptococcus uberis.*

Discussion

In this study, the pathogenicity of two *S. uberis* clinical mastitis isolates with different *in vitro* biofilm formation ability, was studied in an experimental IMI in dairy cows. After inoculation, results showed that all the animals challenged with the SU2H strain developed clinical mastitis, while only 3 out of the 4 animals challenged with 0140J did. However, the animal that did not develop clinical mastitis, showed a significant increase in SCC during the

days after the challenge (data not shown), possibly indicating that the infection was efficiently removed by the host's immune response. Nearly the same results were reported by Hill (1988) for an intramammary challenge with the 0140J strain that caused infection and disease in 16 out of 18 challenged quarters and by De Greeff *et al.* (2013) in 3 out of the 4 infected quarters. Moreover, the 0140J strain, as a well-known challenge strain, had been used in other efficacy trials. For instance, Leight *et al.* (1999) found similar results in terms of incidence of clinical mastitis after challenge in the control group. Therefore, the results obtained in the present study are in concordance with the literature in terms of incidence for the 0140J strain.

Furthermore, the *in vitro/in vivo* generation time and bacteriological count were assessed in order to analyse the dynamics of the infection. The *in vivo* generation time calculations were based on the number of viable bacteria recovered from milk at each sampling time during the exponential phase of bacterial growth in the mammary gland, which may depend on the combined effects of bacterial replication and bacterial clearance due to host defences and daily milking. The results showed that there were no differences in the *in vitro* generation time between strains and, that in both cases, it was lower than the *in vivo* generation time. This is in accordance with Gilbert *et al.* (1987), who stated that in many infections microorganisms grow more slowly than in the laboratory due to nutritional requirements. In contrast, the biofilm forming SU2H strain exhibited a significantly lower *in vivo* generation time compared to the 0140J strain, indicating that the SU2H strain has a better ability to survive in the mammary

gland as it needed less time than the 0140J strain to multiplicate, at least in the early days after the challenge. On the other hand, the bacterial profile displayed once both infections reached the stationary phase was completely different. The biofilm forming strain (SU2H) showed significantly low titers of viable bacteria up to the end of the study, whereas for the non-biofilm forming strain 0140J the titers remained stable and significantly higher than those of the SU2H isolate. This could indicate that each strain has a different persistence strategy when infused into the mammary gland. With regard to SCC, the group infected with the 0140J strain showed significantly higher overall values throughout the study. This may be as a consequence of the higher bacterial cell count observed in the quarters infected with the 0140J strain. A similar increase in bacteriological count and SCC after challenge by the 0140J strain was observed by other authors (Finch *et al.* 1994; Hill *et al.* 1994; Leigh *et al.* 1999; Leigh *et al.* 2010). In these previous studies, the bacteriological count and SCC described similar peak values of around 6 - 7 logCFU/ml or logCells/ml in the control groups as those observed for the animals infected with the 0140J strain in the present study. With regard to milk production, despite no overall significant reduction being recorded in animals infected with the 0140J strain, a significant reduction ($P < 0.05$) was found the last week of the study. Finally, there were no statistical differences ($P = 0.13$) in the clinical signs of mastitis in infected quarters between the two groups but quarters infected with 0140J showed numerical greater overall results. Therefore, it can be said that the comparison of each parameter between and within the groups showed different and consistent

profiles respectively, confirming that the different pathogenicity observed between groups was due to the strains, thereby ruling out the potential for the results to be due to individual animal variability.

These findings could be the starting point for an investigation into the virulence factors that could be involved in the different infection kinetics observed in the SU2H and 0140J strains. However, until further studies have been performed, our hypothesis, based on the *in vitro* biofilm forming phenotype observed, is that the biofilm forming SU2H strain was able to persist in the mammary gland without exhibiting high levels of bacterial replication, because it may be more able to adhere to the epithelial cells and survive in a biofilm growth mode, while the non-biofilm forming 0140J strain may need higher levels of replication to survive and persist in a planktonic growth mode. However, as the 0140J strain's ability to form a biofilm *in vitro* was previously shown to be dependent on culture conditions (Varhimo, 2011), it cannot be assumed that 0140J is not forming a biofilm *in vivo* after the challenge. Therefore, further investigations are needed in order to determine the *in vivo* biofilm formation capability of the SU2H and 0140J strains after an IMI. Moreover, more clinical isolates should be analysed in order to confirm the relationship between *in vitro* biofilm formation and the dynamics of the IMI. On the other hand, it cannot be ruled out that other virulence factors, not related with the biofilm forming phenotype, could be involved in the different infection kinetics observed between the SU2H and 0140J strains.

So, taken together, the results indicate that the biofilm forming and non-biofilm forming *S. uberis* strains tested in this study exhibit clear differences in the course of the infection, in terms of generation time, bacterial count, SCC, temperature and milk production, suggesting a different colonisation and persistence strategy in the mammary gland. However, further studies are crucial for understanding the dynamics and pathogenesis of IMI with these strains of *S. uberis*.

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Capítol 2. Probing vaccine antigens against bovine mastitis caused by *Streptococcus uberis*

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Probing vaccine antigens against bovine mastitis caused by *Streptococcus uberis*

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ABSTRACT

Streptococcus uberis is a worldwide pathogen that causes intramammary infections in dairy cattle. Because virulence factors determining the pathogenicity of *S. uberis* have not been clearly identified so far, a commercial vaccine is not yet available. Different *S. uberis* strains have the ability to form biofilm *in vitro*, although the association of this kind of growth with the development of mastitis is unknown. The objective of this study was to evaluate the potential use as vaccine antigens of proteins from *S. uberis* biofilms, previously identified by proteomic and immunological analyses. The capability of eliciting a protective immune response by targeted candidates was assayed on a murine model. Sera from rabbits immunized with *S. uberis* biofilm preparations and a convalescent cow intra-mammary infected with *S. uberis* were probed against cell wall proteins from biofilm and planktonic cells previously separated by two-dimensional gel electrophoresis. Using rabbit immunized serum, two proteins were found to be up-regulated in biofilm cells as compared to planktonic cells; when serum from the convalescent cow was used, up to sixteen biofilm proteins were detected. From these proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-biphosphate aldolase (FBA), and elongation factor Ts (EFTs) were chosen to be tested as vaccine antigen candidates. For this purpose, different groups of mice were immunized with the three recombinant-expressed proteins (each one formulated separately in a vaccine), and thereafter intraperitoneally challenged with *S. uberis*. The three proteins induced specific IgG antibodies, but a significant reduction of mortality was only observed in the groups of mice vaccinated with FBA or EFTs. These results suggest that FBA and EFTs might be considered as strong antigenic candidates for a vaccine against *S. uberis* bovine mastitis. Moreover, this is the first study to indicate that also in *S. uberis*, GAPDH, FBA and EFTs, as proteins detected in both cytoplasm and cell wall fractions, can play a second function (*moonlighting*), the latter being particularly involved in the virulence of such a pathogen organism.

1. Introduction

Streptococcus uberis is an important pathogen that causes both clinical and subclinical mastitis in dairy cattle, which leads to economic losses estimated at US$35 billion in the worldwide dairy industry [1]. Recent studies have described differences in virulence among *S. uberis* isolates, classifying the strains into host-adapted, characterized by the chronicity of the infections and the contagious transmission within a herd, and non-adapted strains, related with transient infections of environmental origin [2]. The resistance against macrophage killing, the first defence against intramammary infections, and the ability to adhere to the mammary epithelial cells, are *in vitro* traits of *S. uberis* that correlate with its virulence *in vivo* [3]. The capacity to form biofilms has been described by some authors as an important virulence factor in several bacterial pathogens of veterinary relevance [4]. Bacteria arranged in multilayers and enclosed in the extracellular biofilm matrix become resistant to antimicrobial agents and to the host immune system by impairing the action of phagocytic cells [5]. Such characteristics facilitate the adhesion to and colonization of the mammary gland epithelium by biofilm-forming pathogens, which ultimately allow the establishment of persistent infections [6]. It has been reported for *Staphylococcus aureus* that strains isolated from the mammary gland are more capable of forming biofilm *in vitro* than strains isolated from extra-mammary sources such as teat skin and milking unit liners [7]. Concerning *S. uberis*, Varhimo et al. [8]

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0264-410X/

demonstrated that strains isolated from milk were able to produce biofilm *in vitro*, although this characteristic has not been conclusively associated with virulence differences *in vivo* [3]. A list of putative genes related to virulence was reported for *S. uberis* [9]: *SUB1111* (Fibronectin-binding protein), *SUB1273* (Hemolysin like protein), *SUB1154* (C5a peptidase precursor), *SUB0881* (Sortase A), *SUB0145* (Lactoferrin binding protein), *SUB1095* (Collagen like surface-anchored protein), *SUB1635* (SUAM protein) and *SUB1785* (PauA Streptokinase precursor). However, Hossain and colleagues [10] have probed by comparative genomics analysis that these genes are conserved in both clinical and non-clinical strains, not allowing the differentiation between virulent and avirulent isolates.

Currently, there are no commercially available vaccines, and measures to control *S. uberis* mastitis are limited to the implementation of good-management practices (*i.e.*, teat disinfection, antibiotic therapy and culling of chronically infected cows), that have a rather scarce effect on the prevalence and incidence of the disease [11]. In the experimental design of vaccines against *S. uberis* different approaches have been assayed in cows, but none has demonstrated a complete efficacy upon challenge by heterologous intramammary infection. Protection of the mammary gland against infection with a homologous *S. uberis* strain was achieved by multiple intramammary vaccinations with killed bacterial cells [12]. The same authors observed that a combined vaccination with live *S. uberis*, subcutaneously administered, and intramammary infusion of a bacterial extract did protect against a homologous challenge. Unfortunately, this method was found to be less effective when a heterologous strain was used for challenging [13]. A subunit vaccine based on the bovine plasminogen activator (PauA) from *S. uberis* conferred a 37.5–62.5% heterologous protection from clinical disease [14]. Another subunit vaccine, based on the recombinant Gap C protein from *S. uberis* [15], showed protection results that were subsequently brought into question by Leigh [16]. The *S. uberis* adhesion molecule (SUAM) [17] induces a humoral response with production of antibodies that reduce the adherence and the internalization of *S. uberis* into bovine mammary epithelial cells [18]. The same authors demonstrated that intramammary infusion of *S. uberis*, opsonized with antibodies against SUAM, reduced both bacterial counts and clinical symptoms of bovine mastitis [19].

To identify factors involved in *S. uberis* biofilm formation, a differential protein expression analysis under planktonic and biofilm growth conditions was performed by Crowley et al. [20]. This work suggests that proteins predicted to be involved in glutamine transport (SUB1152), adhesion (SUB0837 and SUB1152), internalization (SUB1212), and sugar metabolism (SUB0135, SUB0235, SUB0161 and SUB0750) participate in the early phase of biofilm growth. However, the immunogenicity of these proteins was not tested. In the present study, we have identified by means of proteomic several cell wall proteins from *S. uberis* biofilms, strongly reacting against a serum from an immunized rabbit or from a mastitis-convalescent cow. Three out the seventeen identified proteins (*i.e.*, glyceraldehyde-3-phosphate dehydrogenase, GAPDH; fructose biphosphate aldolase, FBA; elongation factor Ts, EFTs), were recombinantly expressed and used to probe their immunogenic and protective potential in a murine experimental model.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All the *S. uberis* strains used in this study were clinical bovine mastitis isolates previously characterized as strong biofilm producers according to the method of Stepanovic et al. [21]. For the cell wall proteins preparation, *S. uberis* SU1H (Hipra strains collection) was grown adhered to the surface of 225 cm^2 cell culture flasks with filter caps (Nunc), using Tryptic Soy Broth supplemented with 0.5% Yeast Extract (TSB-0.5%YE) as culture medium and statically incubated at 37 °C in 5% CO_2 for 48 h. Alternatively, *S. uberis* SU1H strain was grown on Agar-Milk (Sigma) solid cultures at 37 °C in 5% CO_2 for 20–24 h. Planktonic cultures of SU1H strain were grown in suspension by shaking at 37 °C for 20–24 h in TSB-0.5%YE medium. To prepare the inocula for the experimental infection of cows and mice, *S. uberis* SU2H strain (Hipra strains collection) was grown on Tryptic Soy Agar supplemented with 0.5% Yeast Extract (TSA-0.5%YE) at 37 °C in 5% CO_2 for 20–24 h. *S. uberis* SU3H strain (Hipra strains collection), grown in TSA-0.5%YE, was used to produce the hyperimmune sera from rabbits.

2.2. Extraction of proteins from bacterial cells

Cell wall-associated proteins were extracted as previously described by Cole et al. [22]. Basically, the extraction process used mutanolysin, an *N*-acetylmuramidase, to gently solubilize cell wall-associated proteins. The cells were first harvested by centrifugation (5000*g* for 20 min at 4 °C), washed with buffer and resuspended in a solution containing lysozyme (100 mg/ml) and mutanolysin (217 U/ml) (Sigma). Following incubation at 37 °C, the sample was centrifuged and the supernatant containing the soluble cell wall-associated (CW fraction) proteins was collected. The pellet, containing cell ghosts, was used when required to extract cytoplasmic proteins (CM-CP fraction) as follows. The pellet of cell ghosts was resuspended in 50 mM Tris–HCl, 1 mM EDTA, pH 8, buffer containing 1 mM phenylmethylsulfonyl (PMSF solution) and sonicated for 30 s at 130 W (Labsonic U, Standard Probe 5T, B Braun). Lysed cell ghosts were centrifuged at 14,000*g* for 15 min at 4 °C and the supernatant, containing the CM-CP protein fraction, was recovered. Cell wall and CM-CP protein extractions were also performed with *S. uberis* cells previously incubated for 1 h at 37 °C in a solution containing 2 mg/ml proteinase K (Sigma); then, bacterial cells were washed twice with PMSF solution and processed as described above.

2.3. Preparation of serums for the proteomic analysis

Hyperimmune serum against *S. uberis* biofilm cells was produced in New Zealand white rabbits as follows. Subcutaneous immunizations were done with 10^{10} bacterial cells of SU3H strain inactivated with 0.1% formaldehyde in phosphate-buffered saline, pH 7.4 (PBS). Rabbits received a total of three doses of killed bacterial cells emulsified in Freund's complete adjuvant (Sigma) in the first dose, incomplete adjuvant in the second dose (14 days thereafter), and finally a third dose without adjuvant (14 days after the second dose). Serum was collected 2 weeks after the third dose.

For the preparation of convalescent serum, a seronegative cow was experimentally infected by intramammary infusion in the right posterior quarter with 5×10^3 CFU of SU2H strain approximately on day 20 of lactation. The infected cow was milked twice daily and clinical signs of mastitis were already observed starting from 24 h post-infection (somatic cell count higher than 200,000 cells/ml, shedding of *S. uberis* in milk, inflammation and abnormal appearance of milk in the infected quarter). Serum was collected 49 days after the intramammary infection.

All the protocols involving animals were approved by the Ethical Committee for Animal Experimentation of Laboratorios Hipra S.A. (Spain), in compliance with Directive 2010/63/EU on the protection of animals used for scientific purposes (adopted on 22 September 2010).

2.4. Two-dimensional gel electrophoresis

Samples were cleaned with 2D Clean-upkit (GE Healthcare) following manufacturer's instructions and using 8 M Urea, 2.5% CHAPS, 2% ASB14, 40 mM Tris–HCl as a rehydration buffer. First-dimension isoelectric focusing was carried out on immobilized 7-cm strips (GE Healthcare) with a lineal pH gradient from 4 to 7 in an IPG Phor3. An amount of 25 μg total protein was mixed with 1.6 μl of 0.5 M DTT and 0.25 μl of IPG buffer (GE Healthcare) in a final volume of 50 μl. Samples were loaded in the strips and focused to 15 kV h. After focusing, strips were incubated for 15 min with an equilibration solution (6 M Urea, 2% SDS, 50 mM Tris–HCl, 0.02% bromophenol blue) containing DTT at 10 mg/ml and then for other 15 min with equilibration solution containing iodoacetamide at 25 mg/ml. For the second dimension, the strip was applied to a 12% acrylamide gel (Bio-Rad) and electrophoresed for 80 min at 15 mA. Gels were stained with Instant blue (Fischer Scientific) or PlusOne silver staining kit (GE Healthcare). EZ Run (Fisher bioreagents) was used as protein ladder following the manufacturer's instructions.

2.5. Western blotting

Proteins from one-dimension (12.5% SDS–PAGE) and two-dimension gels (2D-gels) were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Thereafter, PVDF membranes were blocked with 3% bovine serum albumin (BSA) in PBS (pH 7.4) containing 0.05% Tween-20 (PBST) overnight at room temperature. After washing once with PBST, membranes were soaked for 1 h at room temperatures in serum samples diluted in PBST (1:500 for the convalescent and pre-immune cow sera; 1:50–1:100 for the rabbit hyperimmune serum). After washing three times with PBST, the membranes were incubated for 1 h at room temperature alternatively with horseradish peroxidase-labeled protein G (Thermo Scientific) diluted in PBST (1:2000 for convalescent and pre-immune cow sera; 1:10,000 for rabbit hyperimmune serum in SDS–PAGE gels), or goat anti-rabbit horseradish peroxidase-labeled (Thermo Scientific) (1:4000 dilution for rabbit hyperimmune sera in 2D-gels). Finally, membranes were washed three times with PBST and developed using the Horseradish Peroxidase Conjugate Substrate Kit (Bio-Rad).

2.6. Mass spectrometry analysis

Spots picked up from Coomassie stained acrylamide 2D-gels were destained with 50 mM ammonium bicarbonate in a 50% acetonitrile solution. Silver stained 2-D gels were destained for 20 min with a solution containing 100 mM sodium thiosulfate and 30 mM potassium ferricyanide, and exhaustively washed with distilled water prior to in-gel digestion with 20 ng of trypsin (Promega) for 4 h at 37 °C. The resulting peptides were eluted from the gel with 66% acetonitrile and 0.2% of trifluoroacetic acid in water. Mass spectrometry analyses of 0.5 μl of sample premixed with 0.5 μl of α-Cyano-4-hydroxycinnamic acid were carried out in a MALDI-TOF UltrafleXtreme (Bruker Daltonics) by reflector mode at 25 kV. Peptide mass fingerprint identifications (PMF) were performed with the MASCOT search engine (Matrix Science) against the NCBInr database. Scores equivalent to *P*-values higher than 0.05 were not considered as positive.

2.7. Cloning, expression and purification of recombinant proteins

The GAPDH, the FBA and the EFTs proteins were recombinantly produced as follows. Gene fragments corresponding to the NCBI reference sequence WP_015911929.1, WP_012657870.1 and WP_015912046.1 of GAPDH, FBA and EFTs respectively, were synthetically produced with a six His-tag added to the N-terminal of each protein, and cloned into pGTPc608 plasmid (GTP Technology). For the expression of the recombinant proteins (rGAPDH, rFBA and rEFTs), transformed *Escherichia coli* BL21 host cells were cultured in selective GY medium (5% glucose, 1% yeast extract and 50 μg/ml kanamycin) at 37 °C (pGTPc608_GAPDH and pGTPc608_EFTs) or 30 °C (pGTPc608_FBA) until the OD_{600} reached 3.6, 1.95 and 1.14 for pGTPc608_GAPDH, pGTPc608_EFTs and pGTPc608_FBA, respectively. Protein synthesis was induced by adding isopropyl-β-D-thiogalactopyranoside 1 mM final concentration for pGTPc608_GAPDH, pGTPc608_EFTs and 0.1 mM for pGTPc608_FBA. After incubation at 37 °C (or 18 °C for pGTPc608_FBA) for 4 h, bacteria were harvested (15,900g, 15 min) and resuspended in lysis buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 20 mM imidazole, 1 mM DTT). Cells were lysed by sonication, insoluble cell debris removed by centrifugation (15,900g, 30 min, 4 °C), and the supernatants (soluble extract) filtered through a 0.45 μm filter. Soluble extracts were loaded onto Ni^{2+}-Sepharose FF columns (Thermo Scientific) and the recombinant protein eluted by imidazole gradient (50–500 mM). Protein purity was assessed by SDS–PAGE and quantified by image densitometry using a BSA standard.

2.8. Immunization and experimental infection of mice

Female BALB/c mice were purchased from Harlan Interfauna Ibérica (Barcelona, Spain). The mice were fed *ad libitum* in a controlled environment that included light and dark cycles (12 h light:12 h darkness). The protective capability of rGAPDH, rFBA and rEFTs proteins was evaluated on six-seven-week-old BALB/c female mice randomly distributed into six groups, that were subcutaneously vaccinated (day 0) and revaccinated (day 14) with 10 μg of either rGAPDH (group 1, $n = 20$), rEFTs (groups 2, $n = 20$, and 5, $n = 10$), or rFBA (group 4, $n = 10$); groups 3 ($n = 20$) and 6 ($n = 10$), inoculated with PBS, were the non-immunized controls. All the vaccines were formulated with an oil-based adjuvant (Laboratorios Hipra S.A.). Sixteen days after revaccination, each animal was intraperitoneally challenged with $2–4 \times 10^9$ CFU of *S. uberis* SU2H and its clinical status recorded daily during the following seven days (presence or absence of lethargy, ruffled fur and hunched posture). Deaths occurred during the post-challenge period were due to the infection, and surviving mice at the end of the study (seven days post-challenge) were sacrificed.

2.9. Determination of specific antibodies in vaccinated mice

Blood samples were collected from mice for serological analysis on day 14 after revaccination. Serum samples from each group were pooled and assayed in duplicate for specific IgG antibodies against rGAPDH, rFBA and rEFTs by ELISA. Briefly, 96-well plates were coated with 1 μg of the purified recombinant proteins (rGAPDH, rFBA or rEFTs) and blocked with Stabilcoat (SurModics); wells were incubated with diluted serum samples (1:100), and the bound total IgG specific antibodies detected by peroxidase-conjugated protein G (Thermo Scientific); finally, wells were incubated with the chromogenic peroxidase substrate ABTS (SurModics) and the absorbance at 405 nm measured using a microplate reader (TECAN). The mean value of the absorbance was calculated for each serum sample run in duplicate.

2.10. Data analysis

The production of antibodies against recombinant proteins in vaccinated mice was compared with the control groups using a *t*-student test (Microsoft Excel 2010). Mortality of mice in the post-infection period was analyzed by the Kaplan–Meier survival

method to estimate the percentage of surviving individuals at each time-point (IBM SPSS Statistics 22). A chi-squared test (OpenEpi 2.3.1) was used to compare the mortality (%) and morbidity (%) between vaccinated and control groups of mice during the post-challenge period. A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Comparative proteomics of S. uberis biofilm and planktonic cells

To compare cell wall proteins extracted from *S. uberis* under biofilm growth conditions and planktonic culture, samples separated in 2D-gels were detected by immunoblotting using a hyperimmune rabbit serum against whole biofilm cells (Fig. 1). Two proteins were observed specifically in the cell wall extract from biofilm cells and identified as arginine deiminase and FBA (Table 1A).

3.2. Proteomic analysis of immunogenic cell wall proteins from S. uberis biofilm cells

To identify *S. uberis* proteins displaying immunogenicity under a bovine intramammary infection, cell wall proteins extracted from biofilm cells grown in agar-milk were separated in 2D-gels and differentially immunodetected with the serum from a mastitis experimentally infected cow (see Section 2) and the corresponding pre-immune serum as a control (Fig. 2). When both blots were compared, 20 immunoreactive protein spots, corresponding to 16 different proteins, were identified specifically with the convalescent serum (Table 1B).

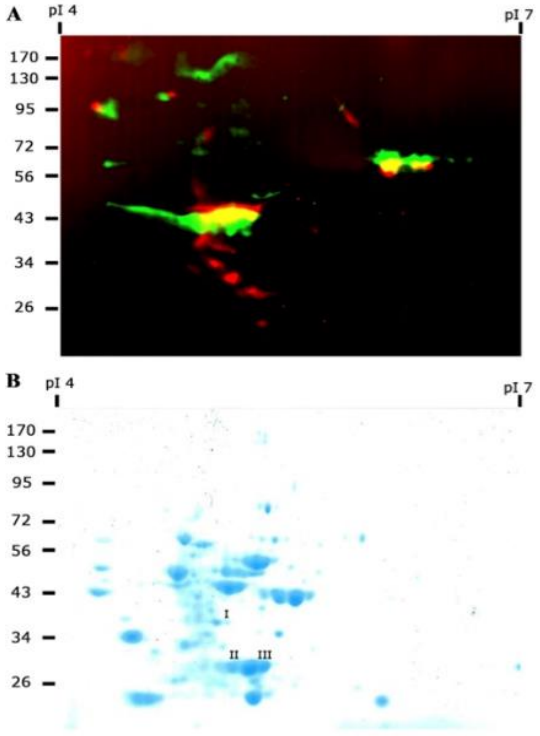

Fig. 1. Identification of biofilm-specific cell wall proteins from *S. uberis*. (A) Western blots overlay of biofilm (red) and planktonic (green) cell wall proteins developed by a hyperimmune rabbit serum against whole biofilm cells. (B) Coomassie blue stained 2D-gel of the biofilm cell wall protein sample. Spots detected only in the biofilm growth condition were picked from the gel and analyzed by MALDI-TOF mass spectrometry. Proteins identified by PMF and MS/MS analyses (Table 1A) are indicated by Roman numbers above the corresponding spot. Numbers at the left side of each panel indicate molecular masses in kDa. The pI range used for the first dimension is indicated.

3.3. Cloning, expression and purification of recombinant FBA, GAPDH and EFTs

From the biofilm proteins specifically identified by rabbit hyperimmune and convalescent cow sera GAPDH, FBA and EFTs were selected as candidate vaccine antigens. The corresponding genes were cloned into the pGTPc608 vector, expressed in *E. coli* as histidine-tagged recombinant proteins (rGAPDH, rFBA and rEFTs) and purified from soluble extracts obtained by sonication (see Section 2). The production yield of rGAPDH, rFBA and rEFTs was estimated at 90, 0.3 and 55 mg, respectively, per liter of culture after purification. Fig. 3 shows the electrophoretic analysis of purified samples by one-step purification using Ni^{2+}-affinity chromatography. Whereas rGAPDH and rEFTs purified samples appear to be homogeneous under Coomassie stain, rFBA shows to be partially purified, although the apparent molecular mass of the more intense protein band does correspond to that expected for rFBA (32,088 Da).

3.4. Immunological response to vaccination with rGAPDH, rFBA, and rEFTs and protective efficacy in a murine infection model

BALB/c mice were immunized with rGAPDH (group 1), rEFTs (groups 2 and 5), and rFBA (group 4). Significant production of specific IgG was observed in all groups of mice vaccinated with rGAPDH, rFBA, and rEFTs as compared to the non-immunized groups 3 and 6 (Fig. 4). At sixteen days post-revaccination, mice were intraperitoneally infected, and clinical signs (lethargy, ruffled fur and/or hunched posture) were observed in all the animals at eight hours after challenge. Significant differences in the percentage of survival were observed between groups ($P < 0.05$) in the post-challenge period. Specifically, a significant ($P < 0.05$) decrease in mortality was observed at 24 h post-infection in mice vaccinated with rFBA (30% mortality for group 4 *vs.* 80% mortality for group 6,) and rEFTs (70% and 40% mortality for groups 2 and 5 *vs.* 95% and 80% mortality for groups 3 and 6). In contrast, vaccination with rGAPDH did not reduce the mortality observed in control animals at 24 h post-infection (90% mortality for group 1 *vs.* 95% mortality for group 3). At the end of the observation period, the mortality rate did not differ between vaccinated and control groups, ranging from 90% to 100% (Fig. 5).

3.5. Cellular location of GAPDH, FBA and EFTs

In order to investigate the cellular location of GAPDH, FBA and EFTs, cell wall associated proteins and CM-CP fraction were extracted from *S. uberis* SU01H cells grown on TSA-0.5YE and each fraction was analyzed by western blot using specific antisera from mice immunized with GAPDH, FBA or EFTs. The three proteins were detected in both cell wall and CM-CP fractions. When *S. uberis* cells were treated with proteinase K prior to the cellular fractionation, GAPDH and FBA were still detected in the CM-CP extract, but absent in the cell wall fraction (Fig. 6). Even the EFTs can, albeit slightly, be observed in the CM-CP fraction from treated *S. uberis* cells, while clearly not detected in the cell wall fraction from treated cells (Fig. 6). These results demonstrate that GAPDH, FBA and EFTs are cytoplasmic proteins that are also found associated to the cell wall of *S. uberis*.

Table 1
Cell wall proteins from *S. uberis* biofilm identified by (A) serum from rabbit immunized with whole biofilm cells and (B) serum from *S. uberis* infected cow (see Section 2).

Spot number	Sequence identifiers	NCBInr matched protein	Theoretical MW (Da)	MASCOT score	Sequence coverage (%)
A					
I	gi|222152511	Arginine deiminase	46,306	92	22.9
II	gi|222153454	Fructose-bisphosphate aldolase	31,015	150*	5.4*
III	gi|222153454	Fructose-bisphosphate aldolase	31,015	113	44.4
B					
1, 2	gi|79102116	Adhesion protein	97,733	66/51*	13/1.21*
3	gi|222153553	Alpha-glycerophosphate oxidase	67,711	133	33.2
4	gi|222153727	Peptide binding protein	60,249	140	58.3
5	gi|222153835	Dipeptidase	55,451	162	56.4
6, 7	gi|222153091	Formate-tetrahydrofolate ligase	59,753	162/100	33.5/25.7
8	gi|222153954	Inosine 5′-monophosphate dehydrogenase	52,844	246	45.8
9	gi|222153531	Nicotinate phosphoribosyltransferase	55,512	159	23.8
10	gi|222152837	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	45,455	126	35.2
11, 12	TRMFO_STRU0	Methylenetetrahydrofolate-tRNA-(uracil-5-)-methyltransferase	49,179	122/167	28.8/37.4
13	GLMU_STRU0	Bifunctional GlmU protein	49,716	129	26.9
14	PGK_STRU0	Phosphoglycerate kinase	42,308	114	42.7
14, 15	ARCA_STRU0	Arginine deiminase	46,306	113/181	38.8
16	EFTS_STRU0	Elongation factor Ts	37,505	145	39.9
17	gi|222152239	Tagatose 1,6-diphosphate aldolase	36,312	88	33.1
18, 19, 20	gi|222153732	Glyceraldehyde-3-phosphate dehydrogenase	35,998	111/152/85.8	41.1/47.0/42.9
20	gi|222153453	Ornithine carbamoyltransferase	37,950	136	48

Sequence identifiers from GenBank or UniprotKB databases.
* Data obtained from MS/MS analyses when PMF score was under the significance threshold (P-value < 0.05).

4. Discussion

In this study, cell wall proteins from *S. uberis* biofilm cells were analyzed to target antigen candidates for a vaccine against bovine mastitis. In a first experimental approach, the arginine deiminase and the FBA, were identified in protein samples from biofilm cells but not from planktonic cultures. In a second approach, sixteen proteins from biofilm cells proved to be highly immunogenic in a bovine intramammary infection induced experimentally (Table 1). A few of these proteins have already been reported as *S. uberis* cell wall associated proteins: the FBA was differentially expressed under biofilm growth conditions [20]; the adhesion protein SUAM is involved in the internalization of the bacterium into bovine mammary epithelial cells [17]; the protective capabilities against bovine mastitis of a recombinant GAPDH was also assayed by Fontaine et al. [15]. With the exception of the adhesion protein and the peptide binding protein (an ABC transporter substrate-binding protein), the proteins identified in the present study were known to be of cytosolic location rather than associated to the cell surface in *S. uberis*. However, several proteins from Table 1 clearly belong to the currently growing group of moonlighting proteins that exhibit an alternative function frequently related to virulence, as reported in other bacterial pathogens [23]. The arginine deiminase and the EFTs were identified in an immunoproteomic analysis of *Streptococcus suis* [24] and *Streptococcus pyogenes* [25] surface proteins. The FBA was detected in the cell wall of *S. suis* biofilm cells [26], as well as in the surface of *Streptococcus pneumoniae* [27] and *S. pyogenes* [25], and it was reported to function as an adhesin in the surface of *Neisseria meningitidis* [28]. The inosine 5′-monophosphate dehydrogenase was described as a plasminogen binding protein in the surface of *S. aureus* [29]. The UDP-N-acetylglucosamine 1-carboxyvinyltransferase, the ornithine carbamoyltransferase and GAPDH were described as cell wall proteins with an increased expression level in *S. suis* biofilm cells compared to planktonic cells [26]. Moreover, GAPDH has been found associated with the bacterial cell surface acting as: transferrin and plasminogen binding in *S. aureus* and *Staphylococcus epidermidis*; fibronectin binding in *S. pyogenes*; adhesin in *N. meningitidis*; plasminogen binding in *Streptococcus anginosus*, *Streptococcus oralis*, *Streptococcus agalactiae*, *S. suis* and *S. pneumoniae* [29]. The ornithine carbamoyltransferase has also been identified as a fibronectin binding protein in *S. epidermidis* [29] and has been detected in the surface of *S. pyogenes* [25]. A plasminogen binding function was attributed as well to the phosphoglycerate kinase localized in the cell surface of *S. anginosus*, *S. oralis* and *S. agalactiae* [29]. The phosphoglycerate kinase, tagatose 1,6-diphosphate aldolase and dipeptidase have been found associated to the cell wall of *S. pyogenes* [25]. Finally, the first demonstration of elongation factors associated with the cell surface in streptococci was reported by Wilkins et al. [30]. For the other proteins identified in the present study (*i.e.*, alpha-glycerophosphate oxidase, formate-tetrahydrofolate ligase, nicotamine phosphoribosyltransferase, methylenetetrahydrofolate-tRNA-(uracil-5-)-methyltransferase and bifunctional GlmU protein), no moonlighting function or cell surface location have been previously described, and it may be of interest to gain insight into their likely alternative role.

Since the moonlighting function of a protein is normally carried out in a cell location different from that of the "canonical" activity, we have determined where GAPDH, FBA, and EFTs are located. The results obtained by western blot of cell wall (CW) and membrane-cytoplasmic (CM-CP) protein fractions, using specific murine antisera, show that the GAPDH, EFTs and FBA proteins are found associated to the cell wall and membrane-cytoplasm compartments (Fig. 6).

Taking into account the data as a whole, (*i.e.*, bibliographic, cellular location, high immunoreactivity, and moonlighting activity related to virulence), we have chosen GAPDH, FBA and EFTs proteins as potential antigens for a vaccine against mastitis caused by *S. uberis*. Moreover, we have considered the use of a minor animal species to test the immunogenicity and efficacy of the vaccine candidates in the screening phase before launching trials in cows. Thus, we have used an intraperitoneal infection model in mice to test individually the selected antigens. Our results indicate that all three proteins elicit a high IgG response in vaccinated mice and that, after infection, only FBA and EFTs conferred a significant increase of the survival at 24 h after challenge. Although no significant differences between groups were observed beyond 24 h post-challenge, the results obtained suggest that the immunological

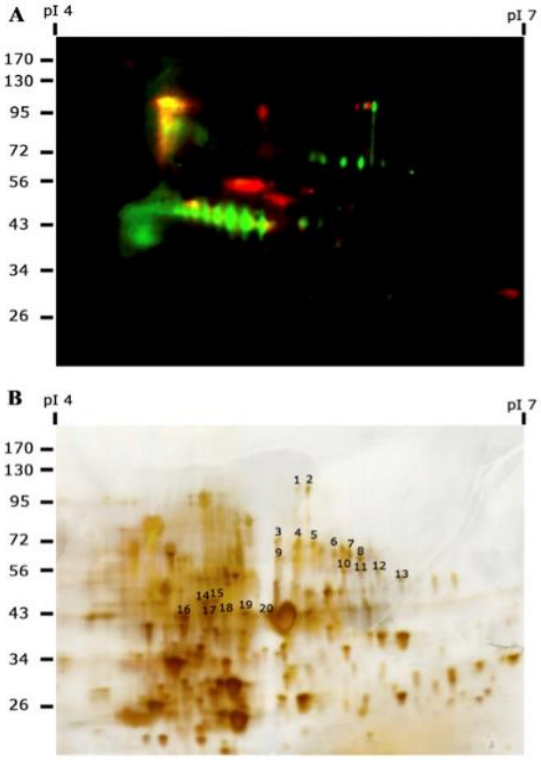

Fig. 2. Identification of immunogenic cell wall proteins from *S. uberis* under a bovine intramammary infection. (A) Overlay of western blots of proteins from *S. uberis* biofilm cells, developed by a bovine pre-immune serum (red) and a mastitis convalescent cow serum (green). (B) Silver stained 2D-gel of the biofilm cells sample. Spots specifically detected by the convalescent cow serum were picked from the gel and analyzed by MALDI-TOF mass spectrometry. Proteins identified by PMF and MS/MS analyses (Table 1B) are indicated by numbers above the corresponding spot. Numbers at the left side of each panel indicate molecular masses in kDa. The pI range used for the first dimension is indicated.

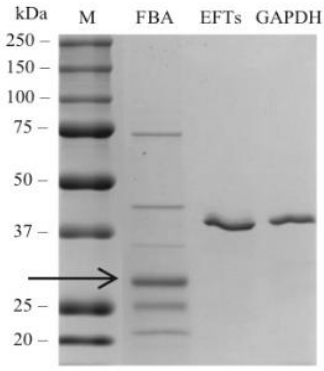

Fig. 3. 12.5% SDS–PAGE analysis of the purified recombinant GAPDH, FBA and EFTs, stained with Coomassie brilliant blue. The arrow indicates the band corresponding to rFBA (32,088 Da). Lane "M" includes molecular weight markers.

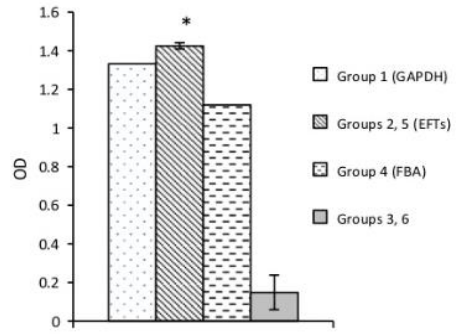

Fig. 4. Specific IgG responses on day 28 in the serum of mice vaccinated (day 0) and revaccinated (day 14) with rGAPDH (group 1), rEFTs (groups 2 and 5) and rBPA (group 4), and non-immunized controls (groups 3 and 6). Serum samples were assayed by ELISA in 96-well plates coated with either rGAPDH (groups 1, 3 and 6), rEFTs (groups 2, 5, 3 and 6) or rFBA (groups 4, 3 and 6). Data are expressed as optical density units (OD). For groups 2 and 5 (immunized with EFTs) and groups 3 and 6 (controls) the mean OD value and the corresponding standard deviation is represented. The statistical analysis was performed only in mice immunized with EFTs, showing significant differences when compared with the control groups (symbol ∗: $P < 0.05$).

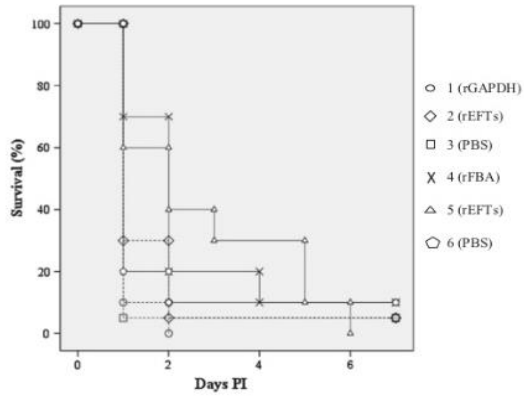

Fig. 5. Survival curves of mice vaccinated with rGAPDH (1), rEFTs (2 and 5), rFBA (4) and non-immunized controls (3 and 6) after *S. uberis* challenge. Survival was monitored daily and is expressed as the percentage (%) of surviving individuals in each group over a period of 7 days post-infection (PI). Dashed lines show data from groups 1, 2 and 3, and solid lines from groups 4, 5 and 6.

response induced by vaccination with FBA and EFTs can confer partial protection by controlling the infection during the first hours, likely interfering with the bacterial multiplication, colonization or/and dissemination. The findings presented here constitute a promising starting point for the development of a vaccine against bovine mastitis. For this purpose, further studies should include a more extensive analysis of the dose/response relationship and the trial of subunits vaccines resulting from the combination of several putative antigens emerging, besides FBA and EFTs, from the set of highly immunoreactive proteins we have identified (Table 1).

Conflict of interest statement

The authors declare no conflicts of interest.

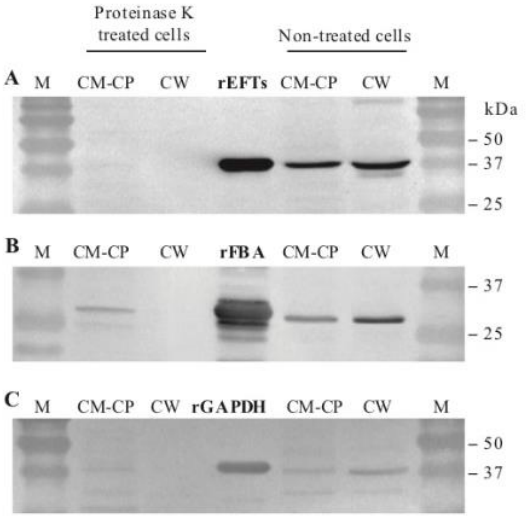

Fig. 6. Cellular location of GAPDH, FBA and EFTs. Western blots of fractions containing cell membrane and cytoplasmic proteins (CM-CP) and cell wall proteins (CW), extracted from proteinase K treated and non-treated cells (20 μg of total protein per lane). Recombinant EFTs, FBA and GAPDH were included as a control. Blots were developed using anti-EFTs (A), anti-FBA (B) and anti-GAPDH (C) murine sera. Lanes "M" include pre-stained molecular weight markers.

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Capítol 3. Study of the efficacy of a *Streptococcus uberis* mastitis vaccine against an experimental intramammary infection with a heterologous strain in dairy cows

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Study of the efficacy of a *Streptococcus uberis* mastitis vaccine against an experimental intramammary infection with a heterologous strain in dairy cows

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ABSTRACT

Streptococcus uberis is a worldwide pathogen that causes intramammary infections in dairy cattle. Nevertheless, commercial vaccines are currently not available and measures to control *S. uberis* mastitis are limited to the implementation of good management practices. The aim of the present study was to evaluate the efficacy of an *S. uberis* subunit vaccine against bovine mastitis (Laboratorios Hipra S.A., Amer, Spain) administered precalving against an experimental intramammary challenge with a heterologous *S. uberis* strain in dairy cows postcalving. With this objective, 25 gestating Holstein-Friesian heifers were randomly assigned to 1 of 2 groups: group 1 (n = 13), vaccinated by intramuscular route with the vaccine, and group 2 (n = 12), vaccinated by intramuscular route with phosphate-buffered saline as a control group. Both groups were immunized 60 and 21 d before the expected parturition date (75 and 36 d before challenge). Fourteen days after calving all cows were challenged by intramammary infusion of 100 colony-forming units of a heterologous *S. uberis* strain in 2 quarters per cow. Then, challenged quarters were monitored for clinical signs of mastitis, bacterial count, and somatic cell count for the following 21 d. Rectal temperature and daily milk yield per cow were also assessed. Results showed that all challenged quarters developed clinical mastitis. Nevertheless, vaccination significantly reduced the clinical signs of mastitis, bacterial count, rectal temperature, and daily milk yield losses after the intramammary infection and significantly increased the number of quarters with no bacterial isolation and somatic cell count <200,000 cells/mL at the end of the study (d 19, 20, and 21 after challenge). To confirm the efficacy of this vaccine, further studies under field conditions are needed.

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Key words: *Streptococcus uberis*, mastitis, vaccine, dairy cow

INTRODUCTION

Streptococcus uberis is one of the major causative pathogens of clinical and subclinical mastitis in dairy cattle. It has been estimated that it is responsible for 14 to 26% of clinical mastitis cases in countries such as Canada, the United States, and the Netherlands and is the main cause of clinical mastitis in New Zealand and Australia (Hogan and Smith, 1997; Wang et al., 1999; McDougall et al., 2007). Historically, *S. uberis* has been classified as an environmental pathogen, as the control of contagious mastitis pathogens with the use of good management practices has a limited effect on the prevalence and incidence of environmental *S. uberis* mastitis (Leigh, 1999). However, in recent years, the development of new molecular typing studies has evidenced that there is also direct transmission and predominance of particular strains in some herds (Zadoks et al., 2003; Davies et al., 2016; Archer et al., 2017). Related to these observations, recent studies described differences in virulence among *S. uberis* isolates in vivo, classifying the strains into host-adapted strains, which may be capable of cow-to-cow transmission, and nonadapted strains, associated with transient infections of environmental origin (Tassi et al., 2013). Afterward, the same group demonstrated that resistance against macrophage killing and the ability to adhere to the mammary epithelial cells were in vitro traits that correlated with *S. uberis* virulence found in vivo (Tassi et al., 2015). Biofilm formation has been described as one of the important virulence factors of some strains of *S. uberis* (Varhimo et al., 2011). Moreover, Crowley et al. (2011) demonstrated that the transition from planktonic to biofilm growth in the *S. uberis* 0140J strain correlated with an upregulation of several gene products that have been shown to be important for pathogenesis.

In parallel with the abovementioned investigations, different approaches have been made in the design of experimental vaccines against *S. uberis* by several au-

thors. For instance, protection of the mammary gland against infection with a homologous *S. uberis* strain was achieved by multiple intramammary vaccinations with killed bacterial cells (Finch et al. 1994). The same authors observed that a combined vaccination with live *S. uberis*, subcutaneously administered, and intramammary infusion of a bacterial extract also protected against a homologous challenge. Unfortunately, this method was less effective when a heterologous strain was used in a challenge (Finch et al., 1997). A subunit vaccine based on the bovine plasminogen activator (PauA) from *S. uberis* conferred 37.5 to 62.5% heterologous protection from clinical disease (Leigh et al., 1999).

Despite the different approaches in the design of experimental vaccines against *S. uberis*, there is still a lack of commercially available vaccines. Therefore, the aim of the present study was to assess the efficacy of a new vaccine against the bovine mastitis caused by *S. uberis*. With this purpose, first, the vaccine strain was selected. Then, the vaccine antigen (**BAC**) was analyzed to identify immunogenic components. Finally, the efficacy of the vaccine administered precalving was tested against an experimental intramammary challenge in dairy cows postcalving.

MATERIALS AND METHODS

Bacterial Strains

Streptococcus uberis strains SU1H, SU2H, SU4H, SU5H, SU6H, SU7H, SU8H, SU9H, and SU10H are bovine mastitis isolates from the Laboratorios Hipra S.A. strain collection. Strains SU1H, SU4H, SU5H, SU6H, SU7H, and SU8H were isolated from clinical cases of bovine mastitis in Spain. Strain SU1H was selected as the vaccine strain based on its high ability to form biofilm in vitro. Strains SU2H, SU9H, and SU10H were isolated from clinical cases of bovine mastitis in the United Kingdom. Strain SU2H was selected as a challenge strain in a previous challenge model study (data not shown). Strain 0140J (ATCC-BAA-854; provided by the American Type Culture Collection, Manassas, VA) is a well-characterized bovine mastitis strain.

Biofilm Formation and Inhibition Assays

Bacterial inoculums for biofilm assays were prepared as follows: lyophilized cultures in glass vials were thawed, suspended with 1 mL of ultrapure sterile water, plated on Columbia III agar with 5% sheep blood (Becton Dickinson, Franklin Lakes, NJ), and incubated overnight at 37°C. After confirmation of viability and purity, 3 single colonies were inoculated in 20 mL of tryptic soy broth (Becton Dickinson) containing 0.5% yeast extract (Becton Dickinson; **TSB+YE**) and incubated at 37°C for 16 h. The ability of *S. uberis* to form biofilm was evaluated using 96-well polystyrene plates (Thermo Fisher Scientific, Waltham, MA) as follows: 16-h cultures of SU1H, SU2H, SU4H, SU5H, SU6H, SU7H, SU8H, SU9H, SU10H, and 0140J were diluted 1:100 in TSB+YE, and 200 μL of the suspensions was used to inoculate 96-well polystyrene plates. Fresh TSB+YE medium without bacterial culture was inoculated as a negative control, and cells were grown for 24 h at 37°C. Then, planktonic suspensions were removed and wells were washed 3 times with PBS and dried for 1 h at 37°C. Cells were heat-fixed and stained with a 0.5% (vol/vol) crystal violet solution (Merck, Darmstadt, Germany) for 10 min at room temperature. The stain was removed and the wells were washed 4 times with 300 μL of PBS. Stained biofilms were diluted in 200 μL of 96% ethanol and then incubated under orbital agitation for 2 h. Finally, the optical density at 595 nm was measured using a microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The experiment was conducted for 3 d using 8 wells in each plate for the culture of 1 strain. The optical density values for 24 wells were obtained for each isolate and used to determine the mean value.

For the biofilm inhibition assay, a 1:2,000 dilution of a vaccinated or unvaccinated sera and a 1:10 or 1:100 dilution of a lipoteichoic acid (**LTA**)-specific monoclonal antibody (Hycult Biotech Inc., Plymouth Meeting, PA) were added to 16-h SU1H cultures used to perform the biofilm formation assay. As a positive control, one 16-h SU1H culture was left without serum or mAb. Once the 96-well polystyrene plates were inoculated, the biofilm formation assay was performed as described above.

Antigen Preparation

The antigen fraction from *S. uberis* was obtained as follows: lyophilized cultures in glass vials of strain SU1H were thawed, suspended with 1 mL of ultrapure sterile water, diluted 1:200 on TSB+YE, and incubated 5 to 6 h at 37°C under stirring. Then, the bacterial suspension was diluted 1:100 in fresh TSB+YE medium and incubated at 37°C for 16 h. After confirmation of viability and purity, overnight cultures were diluted 1:100 in TSB+YE and cultured in cell culture flasks (Nunc, Roskilde, Denmark) at 37°C in 5% CO_2 for 48 h in static conditions. Afterward, adhered biofilm-forming cells were harvested and suspended in deionized water and then autoclaved at 121°C for 45 min and centri-

fuged at 14,000 × *g* for 20 min. Finally, the supernatant was recovered and stored at 4°C as the antigen fraction (BAC).

Western Blotting

Taking into account the extraction method of the antigen, only thermostable components were expected to remain structurally intact after the heat treatment. For this reason, the presence of LTA in the BAC antigen was assessed by Western blot analysis. Test antigen (10 μL) and commercial LTA (50 μg of LTA) from *Streptococcus pyogenes* (ref. L3140; Sigma-Aldrich, St. Louis, MO) as a positive control were mixed with sampling buffer (24 m*M* Tris-HCl, 1% SDS, 5% glycerol, 1.25% β-mercaptoethanol, 0.005% bromophenol blue) and resolved in 10% Mini-Protean TGX precast gels (Bio-Rad, Hercules, CA) using the Mini Protean III electrophoresis system (Bio-Rad). Then, gel was electrotransferred using the Mini Protean III electrophoresis system (Bio-Rad) onto a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). Thereafter, the polyvinylidene difluoride membrane was blocked with 3% BSA in PBS containing 0.05% Tween-20 (**PBST**) overnight at room temperature. After washing with PBST, a 1:150 dilution of an LTA-specific monoclonal antibody (Hycult Biotech Inc.) in PBST was added to the membrane. After 1 h of incubation at 37°C, the membrane was washed in PBST and incubated again with a 1:1,000 dilution of a polyclonal goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc., Cambridgeshire, UK). Then, after washing 3 times with PBST, the membrane was incubated for 5 min in clarity Western ECL substrate (Bio-Rad) and imaged using the ChemiDoc MP imaging system (Bio-Rad).

Cattle and Housing

Twenty-five primigravid Holstein-Friesian dairy heifers in the last trimester of gestation were used in this study. After calving, these heifers were considered cows. The group size (n = 12) was premised upon a 40% reduction in *S. uberis* bacterial count during the 21 d after challenge in vaccinates versus controls. Group sizes were determined using Ene 3.0 (Servei d'Estadistica Aplicada, Universitat Autònoma de Barcelona and the Biometric Department of GlaxoSmithKline, Bellaterra, Spain); estimated log values of bacterial count of cattle becoming infected with *S. uberis* in the vaccinated group and control group were 1.92 and 3.20, respectively, with a power of 0.80 and α of 0.05. An additional heifer was added to the vaccinated group to ensure that 12 vaccinated heifers completed the study. Consequently, 13 heifers were included in the vaccinated group. Heifers were uniquely identified with paired, sequentially numbered ear tags, 1 in each ear. All heifers tested negative for bovine viral diarrhea virus persistent infection by reverse-transcription PCR (data not shown). Cattle were managed under the guidelines and approval of the Animal Care Committee of Laboratorios Hipra S.A. in compliance with Directive 2010/63/EU (European Union, 2010) on the protection of animals used for scientific purposes (granted on September 22, 2010). The experiment was conducted during a 5-mo period in winter and spring on an experimental farm (Gurb, Spain). During the experiment, the average low and high ambient temperature was 4 and 18°C, respectively. Relative humidity ranged from 44% (average daily low) to 89% (average daily high). All heifers were housed together in a single pen with straw bedding and were the only heifers housed in that pen. New straw bedding was added approximately weekly. Heifers were fed with a TMR for cows in gestation and early lactation. Water was offered ad libitum throughout the study. After calving, calves were removed from their dam immediately, and all cows were milked twice daily at 0700 and 1700 h.

Milking Procedures

Before milking, all teats of all cows were cleaned with a dry, single-use disposable paper towel to remove straw or other gross contaminant material. A chlorhexidine solution (OXA digluconate MAST P, Cygyc Industria Quimica S.A., Barcelona, Spain) was applied to each quarter, and after 20 to 30 s each teat was dried using another single-use disposable paper towel. Then, before milk appearance was assessed, all cows were forestripped. A California Mastitis Test paddle was used to assess milk appearance from each quarter. Afterward, quarter milk samples were aseptically collected for antibody analysis, bacterial count, and SCC. Milk samples for bacterial culture and antibody analysis were kept on ice (in the milking parlor) during sampling and then stored at −20°C. A second sample for SCC was collected following the same procedure described above but using nonsterile vials containing a broad-spectrum (bronopol and natamycin combination) preservative tablet (broad spectrum microtabs II; Advanced Instruments Inc., Norwood, MA). These samples were also kept on ice (in the milking parlor) during sampling, and after that they were stored at 4 to 6°C and shipped to a commercial laboratory (Associació Interprofessional Lletera de Catalunya, Cabrils, Spain) twice weekly for analysis by flow cytometry (Fossomatic FC; Foss Iberia S.A., Barcelona, Spain). Time delay between ud-

der preparation and milking was approximately 90 to 120 s when assessment and sampling were performed (see "Sample and Data Collection"); if not, the time delay was approximately 60 s. Milking units were attached just after udder preparation and were manually removed after milking. Afterward, all teats were dipped in iodine-based teat disinfectant. Milk weights were recorded from the in-line weigh jar, and the milking units were manually back-flushed with a dilute chlorhexidine digluconate solution (OXA MAST P, Cygyc Industria Quimica S.A.).

Study Design

All heifers were enrolled in the study between 71 and 77 d before the expected date of parturition. Distribution of these heifers into 2 groups was performed by sorting those heifers in function of the expected date of parturition. Two ranks of heifers were generated: rank 1, with the 13 heifers with the advanced state of gestation, and rank 2, with the remaining 12 heifers. Six heifers of each rank were randomly assigned to the control group, whereas the remaining heifers of each rank were assigned to the vaccinated group using computer software (Excel 2013, Microsoft Corp., Redmond, WA). Consequently, 13 heifers were assigned to the vaccinated group (74.8 ± 0.5 d before expected date of parturition) and the other 12 heifers were assigned to the control group (74.7 ± 0.6 d before expected date of parturition).

Fourteen days after enrollment in the study (between 57 and 63 d before the expected date of parturition), the vaccinated group (n = 13) received the UBAC vaccine (2 mL; Laboratorios Hipra S.A., Amer, Spain), whereas the control group (n = 12) received a PBS solution (2 mL). Both administrations were performed intramuscularly in the neck region. Thirty-nine days later, a second dose of UBAC vaccine or PBS was administered (between 18 and 24 d before the expected date of parturition).

Bacterial count and SCC of quarter milk samples were assessed 5, 2, and 1 d before challenge. After calving, each cow was challenged with *S. uberis* as detailed in the following section. After challenge, mastitis clinical signs, rectal temperature, milk yield, bacterial count, and SCC were monitored for 21 d as described below. Personnel involved in the animal experimental procedures, who gathered data on different parameters, were not aware of the treatment received by each individual heifer because heifers and treatments were randomized by a treatment dispenser who was not involved in gathering data.

Intramammary Challenge

Fourteen days after calving (13.75 ± 1.06 d; d 0 of the study), each cow was challenged with *S. uberis* in the front right and rear right quarters. The averages ± standard errors of the mean of milk yield were 24.7 ± 1.14 and 26.8 ± 1.42 L/d for vaccinated and control cows, respectively. Inoculum was prepared as follows: lyophilized cultures in glass vials of the SU2H strain were thawed and suspended with 1 mL of ultrapure sterile water. Then, they were plated on Columbia III agar with 5% sheep blood and incubated overnight at 37°C. After confirmation of viability and purity, single colonies were seeded in tryptic soy agar (Becton Dickinson) containing 0.5% yeast extract (Becton Dickinson; TSA+YE) and incubated at 37°C for 24 h. Bacterial growth was suspended in chilled PBS, harvested by centrifugation (20 min at 5,000 × *g*, 4°C), and adjusted to 100 cfu/mL. Challenge doses were kept at 4°C in the laboratory. The next morning, the bacterial count result of the SUH2 suspension was checked (108 cfu/mL) and stored on ice in the milking parlor until infusion. Cattle were challenged as previously described (Oliver et al., 2004; Jackson et al., 2012; Kester et al., 2015) with some modifications. After the morning milking, 1 mL of the bacterial suspension was aseptically infused through a sterile plastic cannula into the front right and rear right quarters (216 cfu/cow). Finally, the teats were dipped with an iodine teat dip solution.

Sample and Data Collection

Visual examinations to assess cow demeanor were performed on each cow starting the day before the first vaccination (d −61 of the study) and daily until the end of the study (d 21 of the study). Milk production per cow was recorded twice per day from calving until study completion. Mastitis clinical signs of each quarter and rectal temperatures were monitored on the mornings of d 5, 2, and 1 before challenge (d −5, −2, and −1 of the study); twice per day from challenge to 7 d after challenge (d 0 to 7 of the study); and at the morning milking from 8 to 21 d after challenge (d 8 to 21 of the study). Milk samples for the bacterial and SCC analysis were individually collected from all quarters (infected or not) at the same monitoring times used for assessment of mastitis clinical signs. Blood samples for serological analysis were collected from each cow 60 and 21 d before parturition (d −75 and −36 of the study, respectively), the day of the challenge (d 0), and 21 d after challenge (d 21).

Mastitis clinical signs of all quarters were monitored using a composite score of visual abnormalities in milk

and clinical evaluation of the mammary gland. Clinical scores of abnormalities in milk were performed by visual examination. One score was assigned to each quarter based on a visual 5-point scale, where 0 = normal milk; 1 = presence of small clots or flakes (<5 mm diameter) or slightly yellowish color but normal milk density (normal milk with small clots); 2 = presence of clots >5 mm diameter or yellowish color but normal or slightly affected milk density (normal milk with large clots); 3 = presence of large clots (>10 mm diameter) or marked yellowish color with notable alteration of milk density (coagulated milk or transparent milk); 4 = presence of large or small clots with clearly affected milk color and density; and 5 = bloody milk. Clinical scores of mammary gland were performed by palpation and visual examination and were assigned to each quarter based on a 4-point scale, where 0 = normal appearance of quarter; 1 = pain on palpation, redness and swelling, or sclerosis of <25% of the parenchyma; 2 = pain on palpation, redness and swelling, or sclerosis from 25 to 50% of the parenchyma; 3 = pain on palpation, redness and swelling, or sclerosis from 50 to 75% of the parenchyma; and 4 = pain on palpation, redness and swelling, or sclerosis of >75% of the parenchyma. Then, the composite score of mastitis clinical signs was performed per cow, considering the sum of the scores of milk assessment and mammary gland effects of the 2 challenged quarters per cows.

Bacterial Count and SCC Analysis

Quarter milk samples, collected for the bacteriological analysis, were thawed at 4°C for 12 h. Quantitative bacteriological analysis was performed spreading 100 μL of homogenized milk samples onto Columbia III agar with 5% sheep blood plates by means of the Spiral Plater Eddy Jet 2 (IUL Instruments, Barcelona, Spain). After 24 ± 4 h of incubation at 37 ± 2°C, the identity of *S. uberis* was confirmed by the colony morphology (size, color), Gram stain, and oxidase and catalase reactivity. Colony-forming units per milliliter of each sample, compatible with *S. uberis*, were estimated using the Flash and Go colony counter reader and software (IUL Instruments). Negative samples (with no bacterial isolation) were confirmed again by bacterial culture of 250 μL of homogenized milk samples onto Columbia III agar with 5% sheep blood plates. The determination of SCC was carried out at the referential laboratory, Associació Interprofessional Lletera de Catalunya, by flow cytometry (Fossomatic FC; Foss Iberia S.A.). These results were reported electronically via email attachment.

Antibody Response

Individual serum and milk samples were analyzed in duplicate for specific IgG2 antibodies against LTA by an indirect ELISA. Briefly, 96-well polystyrene plates were coated with commercial LTA from *S. pyogenes* (Sigma-Aldrich) by adding 50 μL of LTA (2.5 μg/mL in carbonate buffer) and incubating the plate for 1 h at 37°C. The wells were washed with PBST and then blocked with 250 μL/well of Stabilcoat (SurModics IVD Inc., Eden Prairie, MN) for 1 h at 37°C. Wells were washed again, and 50 μL of 100-fold diluted serum or 50 μL of whey milk was added to each well. A 100-fold dilution of a positive serum from a previously vaccinated cow was added to the plate as a positive control. This serum was obtained 57 d after the administration of 2 doses of the vaccine. A negative serum from a nonvaccinated heifer was also added to each plate as a negative control. Plates were incubated for 1 h at 37°C. After washing, wells were incubated at 37°C for another hour with 50 μL of monoclonal anti-bovine IgG2 antibody (ref. 9D8P; Thermo Fisher Scientific) at a 1:2,000 dilution in PBST. Plates were washed 3 times, and 50 μL of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; Sigma-Aldrich) was added. Reactions were stopped after 7 min, and the optical density (**OD**) was read at 405 nm. Antibody levels were expressed in relative index percentage (**RIPC**) using the following formula: RIPC = (OD sample − OD negative control)/(OD positive control − OD negative control) × 100.

Calculations and Statistical Analysis

Total mastitis clinical signs per cow were calculated as the sum of the scoring of mastitis clinical signs observed in both challenged quarters per time point assessment. The comparison between the vaccinated and control groups was conducted in all time point assessments using the Wilcoxon rank sum test within PROC NPAR1WAY of SAS (SAS Institute Inc., Cary, NC); therefore, data were not parametric. The area under the curve (**AUC**) of mastitis clinical signs was calculated for each cow using the data gathered from d 0 to d 21; 1 value per cow was obtained. The AUC data of the vaccinated group were compared with those of the control group using a *t*-test within the MIXED procedure of SAS.

Milk samples collected to assess the bacterial count and SCC were analyzed individually at a quarter level. The statistical analysis of bacterial count and SCC was performed using ANOVA in MIXED and GLIMMIX procedures of SAS version 9.4 (SAS Institute Inc.). Quarter values were log-transformed to satisfy normal-

ity assumptions. Treatment, sampling time, the quadratic effect of sampling time, and treatment × sampling time interactions were included as fixed effects. Random effects were considered, including cow-specific random intercept and quarter within cow-specific random intercept, slope, and quadratic sampling time trends. The evolution of bacterial count and SCC had a nonlinear pattern. To allow the fitting of a quadratic model, the analysis was conducted considering times from d 5 onward. Geometric means were estimated for each time period. The data from the bacterial count and SCC of each sampling time per quarter were also used to compare vaccinated and control groups to assess the incidence (percentage) of quarters with no bacterial isolation and SCC <200,000 cells/mL from the afternoon of d 0 to d 21 (per day) using the logistic regression in PROC GLIMMIX of SAS version 9.4.

Milk production recorded from 7 to 1 d before challenge was used to calculate the baseline of milk production of each cow. Then, the percentage of milk production per day was calculated considering the production per day divided by the baseline of each cow. These data per day were analyzed using PROC MIXED of SAS. The model included the fixed effects of treatment, sampling time, and treatment × sampling time interaction and the random effect of each cow within treatment. Compound symmetry was selected as the variance–covariance matrix structure for the increase in milk production data based on best fit according to Schwarz's Bayesian information criterion.

The normal rectal temperatures of cows is considered to range from 38.5 to 39.5°C (Constable et al., 2016). Rectal temperature data were converted into binomial, normal values (≤39.5°C) or abnormal values (>39.5°C). The analysis of this data was performed using the chi-squared test within PROC FREQ in SAS per day.

Data on biofilm formation and inhibition were compared using Student's *t*-test in SAS. Data are shown as mean ± standard deviation. Serum and milk ELISA data within the vaccinated and control groups were compared using Student's *t*-test in SAS. Results are shown as RIPC mean ± standard error of the mean. All values reported are least squares means. Significance was declared at $P \leq 0.05$.

RESULTS

Vaccine Strain Selection Based on Biofilm Formation Ability

Biofilm formation in TSB+YE was confirmed in 7 out of 10 *S. uberis* isolates (SU1H, SU2H, SU4H, SU5H, SU6H, SU8H, and SU9H), as they showed a significantly higher mean optical density compared with the noninoculated wells ($P < 0.05$) in the microplate assay (Figure 1). Furthermore, strain SU1H was selected as the vaccine strain because it described the highest biofilm formation ability, although no significant differences were observed with SU4H and SU6H strains.

Antigen Characterization

Western blot analysis results demonstrated that the BAC antigen contained LTA, as a band was detected with an LTA-specific monoclonal antibody. In addition, serum and milk samples from vaccinated heifers were analyzed by ELISA, demonstrating the immunogenicity of the LTA contained in the BAC antigen. In addition, serum and milk samples from vaccinated heifers were analyzed by ELISA, demonstrating the immunogenicity of the LTA contained in the BAC antigen (Figure 5).

Mastitis Clinical Signs

Before challenge, no cow had mastitis clinical signs or alterations in behavior. After challenge, all cows developed clinical mastitis in the challenged quarters. No signs of clinical mastitis or *S. uberis* isolation were detected in the nonchallenged quarters during the study. As shown in Figure 2A, both groups showed similar increases of clinical signs in the challenged quarters, achieving the highest score value 3 d after experimental infection. From d 4 after challenge until the end of the study (d 21), vaccinated cows showed a significant ($P < 0.05$) reduction in mastitis clinical signs on d 6 a.m., d 7 p.m., d 9 a.m., d 10 a.m., d 11 a.m., d 16 a.m., and d 17 a.m. compared with control cows. The AUC analysis demonstrated that vaccinated cows had a significant reduction in mastitis clinical signs compared with control cows from d 0 to d 21 (2.85 ± 0.22 vs. 3.5 ± 0.17; $P < 0.05$).

Bacterial Count and SCC

Milk samples from nonchallenged quarters were bacteriologically negative to *S. uberis* and other mastitis pathogens throughout the study. Concerning challenged quarters, milk samples collected on d −5, d −2, and d −1 were negative for bacterial culture for *S. uberis* and other mastitis pathogens. After experimental infection, these challenged quarters had a peak of bacterial count on d 2 a.m. and d 3 a.m. in vaccinated and control groups, respectively, as shown in Figure 2B. After that, bacterial count was reduced in both groups until a second peak was observed 6 d after challenge. Vaccinated cows had a significant ($P < 0.05$) reduction in bacterial count from d 15 to d 21 compared with control cows.

In terms of SCC, all cows had SCC <200,000 cells/mL on d −5, d −2, d −1, and d 0. After experimental infection, nonchallenged quarters had <200,000 cells/mL throughout the entire study. Considering the challenged quarters, the experimental infection increased the mean SCC, although no differences were found between groups during the entire study (Figure 2C). With regard to the percentage of challenged quarters with no bacterial isolation and SCC <200,000 cells/mL per day during the study, the vaccinated group described a significantly ($P < 0.05$) greater percentage on d 19 (71.1 vs. 16.7%), d 20 (70.2 vs. 10.8%), and d 21 (66.0 vs. 5.9%) compared with the control group.

Rectal Temperature and Milk Production

After challenge, an increased rectal temperature was observed in all cows. The average temperature reached a peak of 40.1 ± 0.27°C and 40.32 ± 0.30°C at d 2 a.m. in the vaccinated and control groups, respectively. However, regarding the percentage of cows with rectal temperature >39.5°C, no differences between groups were recorded during the first 3 d after challenge (Figure 3). After that, the vaccinated group had a significantly ($P < 0.05$) lower percentage of cows >39.5°C compared with the control group on d 4 a.m., d 5 a.m., d 5 p.m., and d 7 p.m.

No differences were found in milk production of cows between groups before challenge. The averages ± standard errors of the mean of milk yield were 24.7 ± 1.14 L for the vaccinated group and 26.8 ± 1.42 L for the control group. After challenge, milk production decreased in all cows. In vaccinated cows, the mean difference in milk production at baseline decreased, reaching the negative peak at d 2. After that, milk production increased and reached similar values of baseline production on d 8 postchallenge (Figure 4). On the other hand, control cows showed a decrease until d 5, and baseline production was attained on d 18. Vaccinated cows showed significantly ($P < 0.05$) greater values in milk yields compared with the control group on d 9, d 13, d 14, d 15, d 17, and d 21.

Antibody Response

The ELISA results showed that there were no significant differences between groups in the level of serum IgG2 antibodies against LTA before vaccination (d

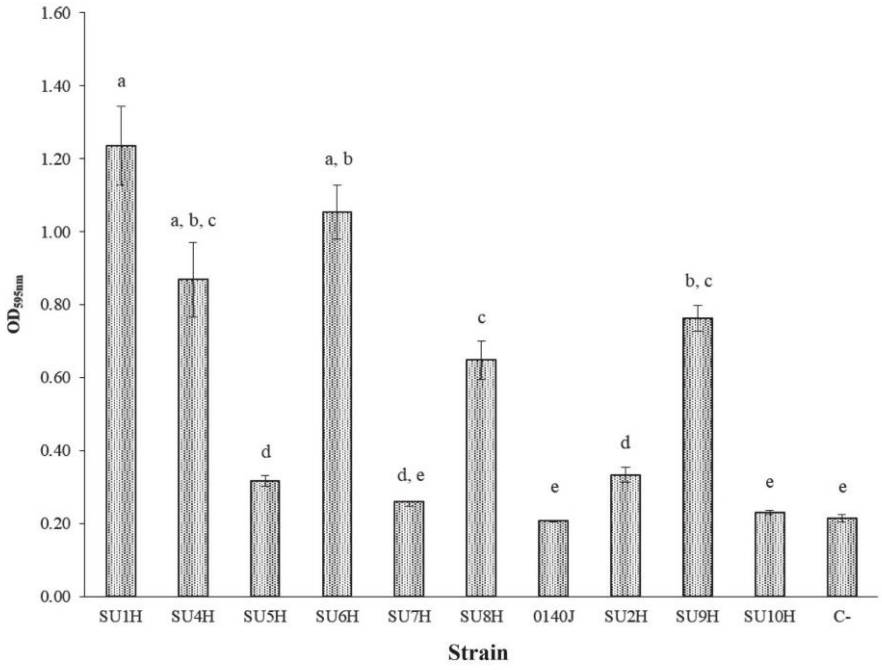

Figure 1. Biofilm formation of *Streptococcus uberis* strains. Biofilm formation was tested in tryptic soy broth + 0.5% yeast extract. Uninoculated media was used as a negative control (C−). Bars represent the average sample optical density at 595 nm (OD_{595nm}) after 24 h of culture at 37°C and SD. The assay was conducted on 3 different days, and for each assay the result was based on the average of 8 wells. Different letters (a–e) indicate significant differences ($P < 0.05$) between strains.

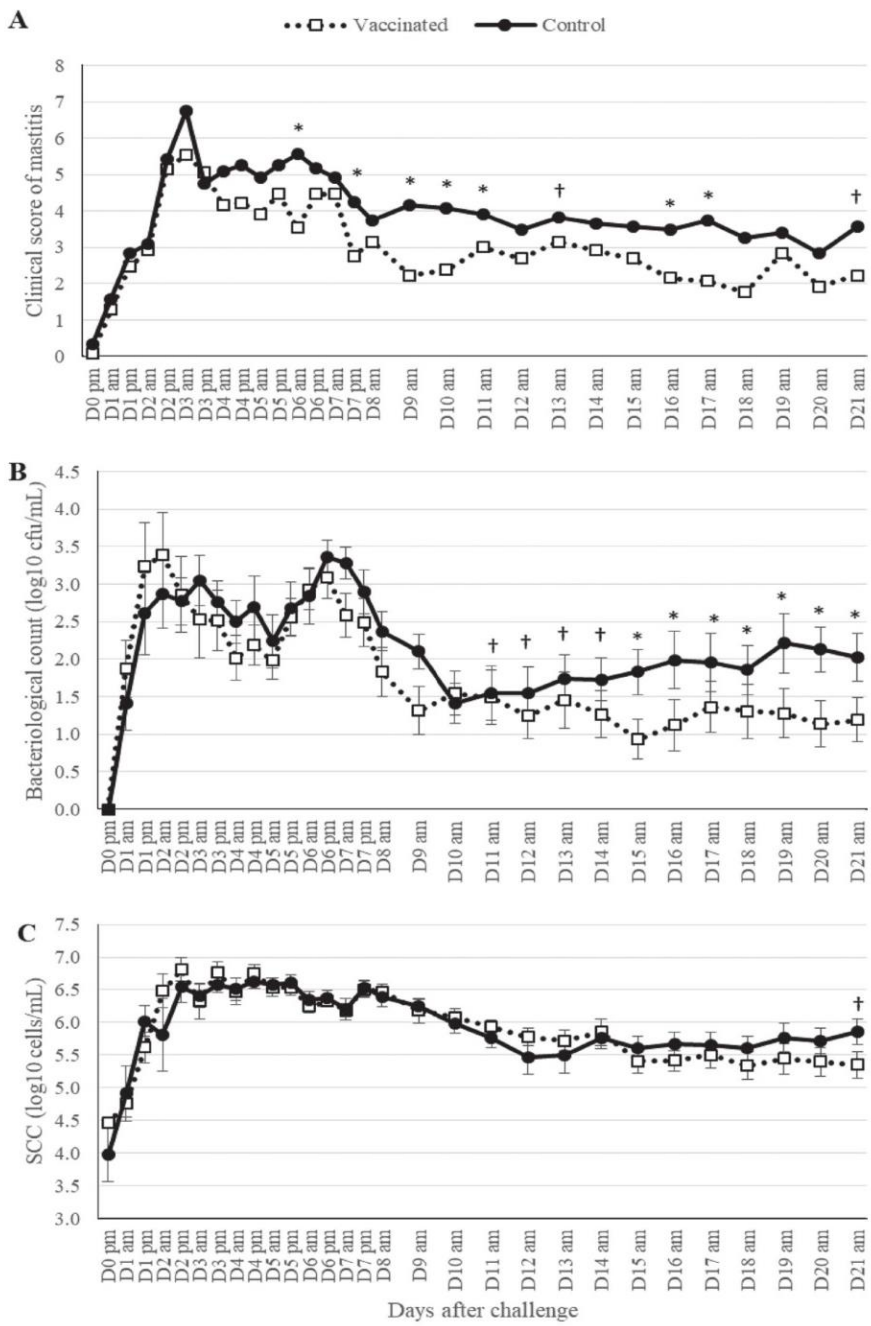

Figure 2. (A) Daily average of clinical mastitis scores of vaccinated and control groups from challenge to 21 d after experimental infection. (B) Geometric means ± SEM of daily bacterial count (log cfu/mL) of vaccinated and control groups from challenge to 21 d after experimental infection. (C) Geometric means ± SEM of daily SCC ($\log_{10}$ cells/mL) of vaccinated and control groups from challenge to 21 d after experimental infection. *Significant differences ($P < 0.05$) between groups; †trends ($0.05 < P \leq 0.10$) between groups.

−75). In response to the first dose of vaccine, the vaccinated group had significantly increased ($P < 0.05$) IgG2 levels against LTA on day −36 compared with the control group, demonstrating the immunogenic activity of LTA contained in the BAC antigen (Figure 5A). Differences between groups were also observed after the second dose of vaccine on d 0 and after the experimental infection on d 21, where higher anti-LTA antibody levels were observed in the vaccinated group compared with the control group ($P < 0.05$). Regarding the antibody response in milk, whey IgG2 levels against LTA in the vaccinated group were higher than those in the control group after the second dose of vaccine on d 0 ($P < 0.05$; Figure 5B). On the other hand, a significant increase in the antibody levels against LTA in serum and milk was found in the control group on d 21 compared with d 0, indicating that the experimental IMI induced an antibody response in control cows.

Biofilm Formation Inhibition Assay

The putative effect on biofilm formation of antibodies generated through vaccination was later evaluated in vitro. First, it was confirmed that the addition of serum (1:2,000) from a vaccinated or unvaccinated cow or an mAb against LTA to an *S. uberis* SU1H culture did not result in any significant difference in their growth (OD_{550nm}) at the end of the incubation period compared with an *S. uberis* SU1H culture without serum or mAb (Figure 6A and Figure 7A, respectively). After that, planktonic cells were removed and cells adhering to the bottom of the microplate were stained. Results showed that wells previously incubated with serum from a vaccinated cow exhibited lower biofilm formation (lower OD_{595nm}) than the wells incubated with a serum from an unvaccinated cow or without serum ($P < 0.05$; Figure 6B). Moreover, wells incubated with a 1:10 dilution of a mAb against LTA showed lower biofilm formation ($P < 0.05$) than the wells incubated with a 1:100 dilution of mAb against LTA or without mAb (Figure 7B). These results indicate that sera and LTA mAb did not have any effect in the total growth (planktonic and biofilm) reached at the end of the incubation. Nevertheless, serum from a vaccinated cow and an mAb against LTA were able to inhibit the adhesion and biofilm formation of *S. uberis* in the bottom of the wells.

DISCUSSION

In this study, the efficacy of a novel *S. uberis* subunit vaccine was evaluated in dairy cows. Cows were vaccinated with a total of 2 doses of the vaccine before calving and challenged with a heterologous strain of *S. uberis* after calving. The vaccine strain SU1H was selected due to its high in vitro biofilm-producing ability, and the vaccine antigen (BAC) was obtained from SU1H biofilm-forming cells and analyzed.

Due to the method of extraction, we believed that only thermostable components could remain functional in the BAC antigen. Therefore, thinking that nonprotein components from the gram-positive bacterial surface could play an important role in the pathogenesis of bacterial infections, we evaluated and confirmed the presence of LTA in the final antigen with an mAb. Sev-

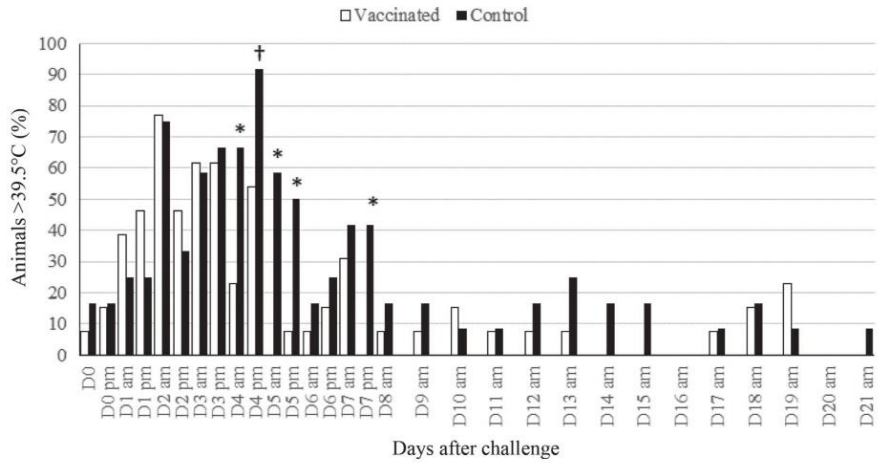

Figure 3. Percentage of cows >39.5°C of vaccinated and control groups from challenge to 21 d after experimental infection. *Significant differences ($P < 0.05$) between groups; †trends ($0.05 < P \leq 0.10$) between groups.

eral authors have previously discussed the relevance of LTA in pathogenesis. Weidenmaier and Peschel (2008) stated that LTA seems to be indispensable for cell viability and important in cell division and in binding cell wall proteins. Moreover, Gross et al. (2001) and Fabretti et al. (2006) demonstrated that LTA mutants of *Staphylococcus aureus* and *Enterococcus faecalis*, respectively, exhibit a reduced primary adhesion that leads to reduced biofilm formation ability in vitro compared with wild type strains. In the same line, our study demonstrated that a specific mAb against LTA was able to reduce ($P < 0.05$) the biofilm-formation ability of *S. uberis* in vitro, suggesting, to our knowledge for the first time, that LTA could be involved in the *S. uberis* biofilm formation in vitro. Therefore, concerning bovine mastitis, the role of LTA during an IMI could be related to the binding of the bacteria to the host epithelial cells in a primary stage, as stated by Czabańska et al. (2012), followed by a step of biofilm formation as a strategy of colonization and persistence in the mammary gland (Melchior et al., 2006).

After challenge, all vaccinated and control cows developed clinical mastitis, indicating that the challenge was successful. However, the vaccinated group showed a significant reduction in the severity of the mastitis clinical signs in the challenged quarters. Furthermore, vaccinated cows showed a significant reduction in the bacterial count in milk of challenged quarters several days after the experimental infection. Regarding SCC, the vaccine did not induce changes in the SCC observed after experimental infection in the challenged quarters compared with the control group. However, when analyzing these data together with the bacterial data, the number of challenged quarters with no bacterial isolation and SCC <200,000 cells/mL becomes significantly ($P < 0.05$) higher in the vaccinated group than in the control group at the end of the study. Rectal temperatures and milk production data were in accordance to those observed for bacterial count. Both groups showed similar results during the 3 d after challenge, but after that an improvement was noticed in the vaccinated group compared with the control group. With regard to rectal temperature, the percentage of cows >39.5°C was significantly greater on d 4 a.m., d 5 a.m., d 5 p.m., and d 7 p.m. in the control group than in the vaccinated group. In terms of cow milk production, a decrease was observed in both groups during the first days after challenge. However, control cows recovered the milk production baseline level 18 d after challenge, whereas vaccinated cows required only 8 d, decreasing milk losses associated with the IMI. Despite these differences, we cannot be sure that milk production levels of the vaccinated group reached the expected milk yield of noninfected cows because a sentinel group was not included in the study.

Data presented in this study demonstrated that vaccination with BAC antigen reduced the clinical symptoms of acute *S. uberis* mastitis caused by ex-

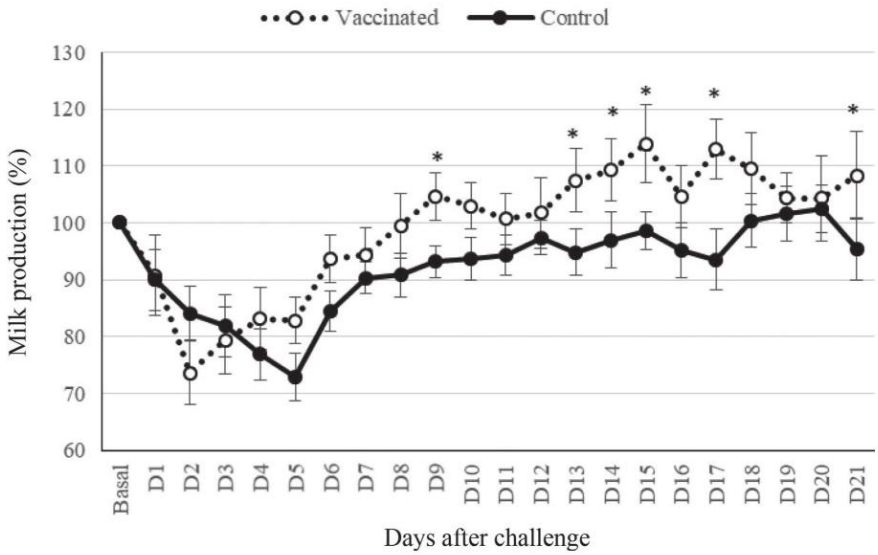

Figure 4. Daily average percentage of milk yield (L) versus prechallenge of vaccinated and control groups from challenge to 21 d after experimental infection. *Significant differences ($P < 0.05$) between groups. Error bars indicate ±SEM.

perimental challenge, possibly associated with lower bacterial concentrations in vaccinated cows. Moreover, this study suggested faster recovery of vaccinated cows after an experimental IMI: the percentage of challenged quarters with no bacterial isolation and SCC <200,000 cells/mL at the end of the study was significantly higher in the vaccinated group, and vaccinated animals recovered their baseline level of milk production earlier compared with the control group. Nevertheless, given the relatively short duration of our trial, it cannot be ensured that the infection was completely cleared in any of the challenged quarters.

Concerning the immune response, our study demonstrated that vaccination with BAC antigen can induce high antibody levels against LTA in serum and milk. Moreover, the activity of these antibodies was tested in vitro, and results showed their ability to reduce biofilm formation in a 96-well microtiter plate. Therefore, according to this result, we hypothesized that protection observed in the present study may be associated with the induction of specific antibodies against *S. uberis* LTA. The presence of these antibodies in milk, when an IMI occurs, could interfere with the binding of the bacteria to the host epithelial cells, as suggested by Czabańska et al. (2012), preventing the subsequent biofilm formation. This could lead to a reduced *S. uberis* colonization rate of the mammary gland, which has been suggested as the mechanism of protection in other

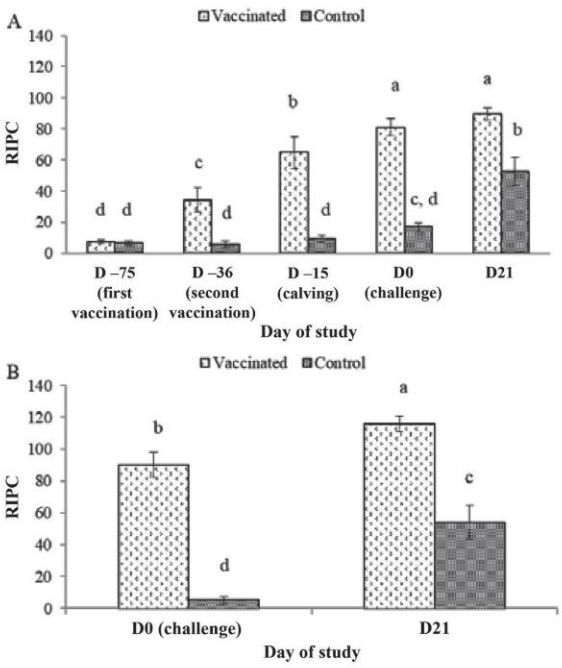

Figure 5. (A) Immunoglobulin G2 responses in sera samples from vaccinated and control heifers measured by indirect ELISA. The first and second doses of the vaccine were given on d −75 and −36, respectively. (B) Immunoglobulin G2 responses in milk samples from vaccinated and control cows measured by an indirect ELISA. Data are shown as the mean of the relative index percentage (RIPC) ± SEM. Different letters (a–d) indicate significant differences ($P < 0.05$) between groups.

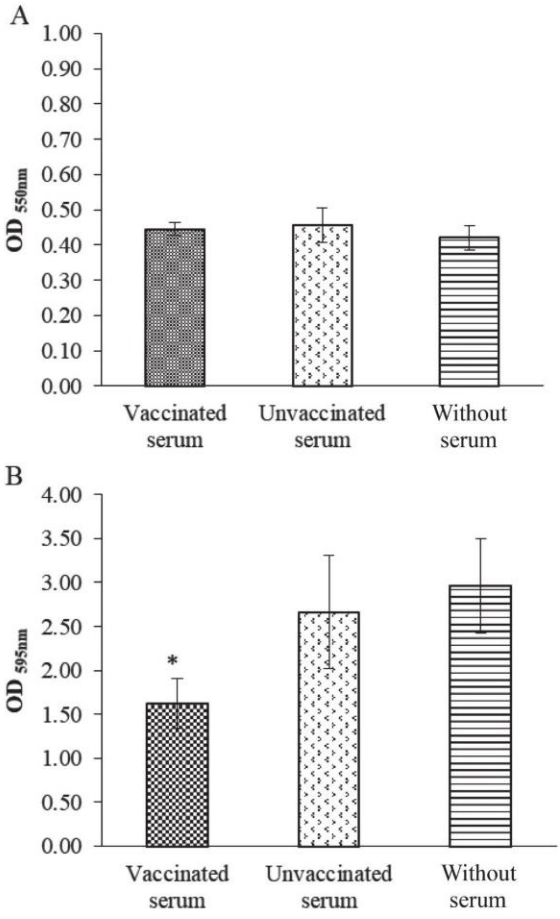

Figure 6. In vitro inhibition of biofilm formation. (A) Growth of *Streptococcus uberis* SU1H in tryptic soy broth + 0.5% yeast extract in the presence of a 1:2,000 dilution of vaccinated and unvaccinated serum and without serum. Bars represent the average optical density at 550 nm (OD_{550nm}) after 24 h of culture at 37°C and SD. (B) Biofilm formation of *S. uberis* SU1H cultures in the presence of vaccinated and unvaccinated serum and without serum. Bars represent the average sample OD_{595nm} after 24 h of culture at 37°C and SD. The assay was conducted on 3 different days, and for each assay the result was based on the average of 8 wells. Significant reductions in in vitro biofilm formation are shown (*$P < 0.05$).

experimental vaccines (Finch, et al., 1994, 1997; Leigh et al., 1999), and a decrease in the persistence of the infection. Moreover, the reduction of bacterial counts in milk of vaccinated cows may also be due to the fact that milk IgG2 antibodies against BAC antigen could opsonize bacterial cells, favoring phagocytosis and clearance by polymorphonuclear neutrophils (Rainard and Riollet, 2003). However, that other components present in the BAC antigen, apart from LTA, could have a key role in the final outcome of the vaccine efficacy should not be ruled out.

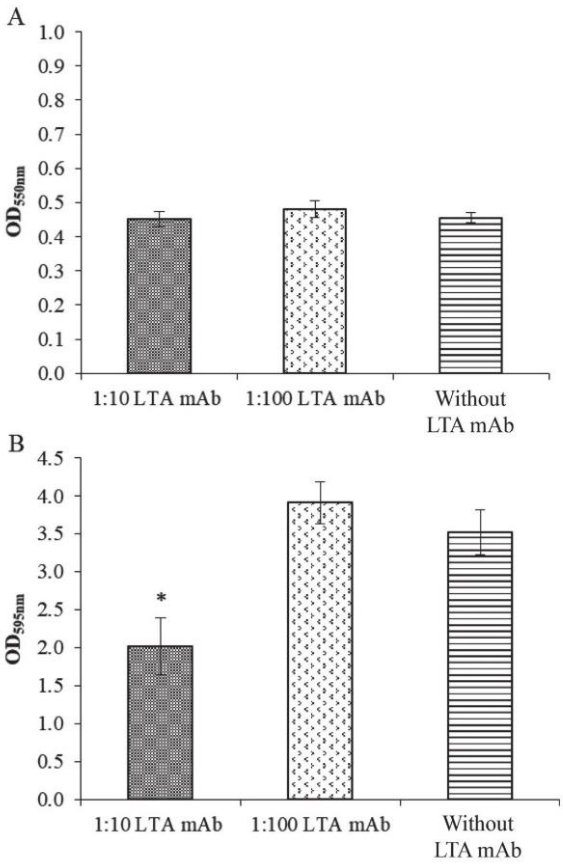

Figure 7. In vitro inhibition of biofilm formation. (A) Growth of *Streptococcus uberis* SU1H in tryptic soy broth + 0.5% yeast extract in the presence of 1:10 and 1:100 dilution of an a monoclonal antibody (mAb) against lipoteichoic acid (LTA) or without mAb. Bars represent the average optical density at 550 nm (OD_{550nm}) after 24 h of culture at 37°C and SD. (B) Biofilm formation of *S. uberis* SU1H cultures in the presence of 1:10 and 1:100 dilution of an mAb against LTA or without mAb. Bars represent the average sample OD_{595nm} after 24 h of culture at 37°C and SD. The assay was conducted on 3 different days, and for each assay the result was based on the average of 8 wells. Significant reductions in in vitro biofilm formation are shown ($^*P < 0.05$).

There are only a few studies in which an experimental *S. uberis* mastitis vaccine has shown some protection upon challenge by heterologous IMI. Finch et al. (1997) tested 2 immunization plans: (1) a subcutaneous prime with a live *S. uberis*, followed by an intramammary booster with a soluble bacterial extract (2 control cows and 3 vaccinated cows), and (2) 2 subcutaneous vaccinations of live *S. uberis* (2 control cows and 4 vaccinated cows). A different approach was tested by Leigh et al. (1999), who used 2 subcutaneous immunizations with a concentrated culture of supernatant containing *S. uberis* plasminogen activator (PauA; 1 control cow and 4 vaccinated cows). In these studies, only limited efficacy in the reduction of the bacterial count in milk (Finch et al., 1997; Leigh et al., 1999) and SCC (Leigh et al., 1999) was observed after challenge. However, these studies used a smaller number of cows, and neither of them reported any statistical analysis supporting the results. In contrast, to our knowledge, this is the first time that a study showed statistically significant differences in mastitis clinical signs, bacterial count, temperature, and milk production, suggesting that vaccination enhanced cows' recovery from clinical mastitis after an acute infection with an heterologous strain.

In conclusion, the results presented in this study demonstrated that intramuscular immunization of dairy heifers with UBAC vaccine during the precalving period reduced the severity of clinical mastitis following experimental challenge of *S. uberis*. Even so, further studies are needed to assess vaccine efficacy under field conditions.

ACKNOWLEDGMENTS

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Discussió general

4. Discussió general

Els objectius principals d'aquesta tesi doctoral s'emmarquen en les fases 1 i 2 del desenvolupament d'una vacuna contra la mamitis bovina causada per *Streptococcus uberis*. Per una banda, s'han identificat i caracteritzat diferents factors de virulència de *S. uberis* relacionats amb la capacitat d'aquest per formar biofilm. Per l'altra, s'ha desenvolupat un model d'infecció experimental en vaques en lactació, amb la finalitat de reproduir la malaltia per avaluar l'eficàcia de vacunes experimentals formulades amb els candidats antigènics identificats i seleccionats durant aquest treball.

Per analitzar l'eficàcia d'una vacuna experimental és imprescindible tenir un bon model experimental en l'animal diana capaç de reproduir la patologia de la manera més fidel possible al que passa en condicions naturals. Per això cal realitzar proves *in vivo* que ens permetin ajustar correctament la via d'administració del patogen, la soca, la dosi infectiva, els mostrejos i la valoració de signes clínics, ja que tots aquests paràmetres influiran en el curs de la infecció i en l'avaluació de l'eficàcia d'una vacuna experimental. Així doncs, el primer objectiu d'aquesta tesi doctoral va consistir en el desenvolupament d'un model d'infecció experimental intramamària capaç de reproduir la mamitis bovina causada per *S. uberis* en vaques en lactació (capítol 1 dels Resultats).

Amb aquest objectiu, es va estudiar la patogenicitat de dues soques virulentes de *S. uberis* aïllades de casos de mamitis clíniques a UK: la soca 0140J, una soca de col·lecció (ATCC-BAA-854) àmpliament caracteritzada i utilitzada en la literatura en proves

d'eficàcia (Finch et al., 1997; Leigh et al., 1999; Ward et al., 2009); i la soca SU2H, un aïllat propi de la col·lecció d'Hipra. En primer lloc, es va determinar la capacitat de les dues soques per formar biofilm *in vitro*. La soca SH2U va resultar ser productora de biofilm, mentre que la soca 0140J no. A continuació, es va realitzar un estudi de patogenicitat, en el qual es van infectar dos grups de vaques en lactació per via intramamària, cadascun amb una de les soques a la mateixa dosi infectiva, en un sol quarteró per animal, i es va seguir l'evolució d'aquests animals diàriament durant 16 dies. Les variables estudiades durant la prova per determinar quina de les dues soques reproduïa millor la mamitis clínica, van ser: el número de quarterons que van desenvolupar mamitis clínica, els signes clínics de mamitis, la producció de llet, els RCS i els recomptes bacteriològics. Després de la infecció experimental, els resultats van demostrar que totes dues soques, administrades a la mateixa dosi per via intramamària, eren capaces de reproduir la mamitis clínica. En el cas de la soca SH2U, en un 100% dels quarterons infectats (4 de 4), mentre que en el cas de la soca 0140J, en un 75% dels quarterons infectats (3 de 4). Aquests mateixos resultats s'havien observat prèviament en altres estudis realitzats pel nostre grup (dades no incloses en aquesta tesi) indicant que la soca SH2U era més eficient a l'hora de causar mamitis clínica. Tanmateix, el grup infectat amb la soca 0140J va mostrar valors més elevats de signes clínics de mamitis, RCS, recompte bacteriològic i pèrdues en la producció de llet, gairebé durant els últims dos terços de l'estudi, suggerint que la capacitat de formar biofilm *in vitro* podria estar relacionada amb una diferent patogenicitat de les soques. De tota manera, només els recomptes

bacteriològics i la producció de llet van ser estadísticament diferents entre els dos grups en alguns dies puntuals. Si comparem els resultats obtinguts per la soca 0140J amb els obtinguts ens altres estudis en IMI amb aquesta soca, podem veure que estan en la mateixa línia. Per exemple, Finch et al. (1997) i Leigh et al. (1999) obtenen resultats similars de recomptes bacteriològics i RCS en els grups control després del challenge, validant els resultats obtinguts en el nostre treball i confirmant que la via d'administració i la dosi escollida en el nostre estudi són adequades per reproduir la malaltia. Finalment, es va seleccionar la soca SH2U com a soca d'infecció per a futures proves d'eficàcia, ja que va ser més eficient a l'hora de reproduir la mamitis clínica (100% dels quarterons infectats) en comparació a la soca 0140J (75% dels quarterons infectats).

Un cop posat a punt el model, el següent pas va ser l'estudi de nous factors de virulència relacionats amb la mamitis bovina causada per *S. uberis*. En aquest treball, vam decidir centrar-nos en el biofilm de *S. uberis* com a font de possibles factors de virulència proteics i no proteics, ja que la seva implicació en la colonització, adhesió i supervivència de diferents espècies patògenes a les cèl·lules MEC és quelcom descrit per diferents autors (Melchior et al., 2006; Elhadidy i Zahran, 2014; Schönborn i Krömker, 2016). Amb l'objectiu, doncs, d'estudiar els components proteics del biofilm de *S. uberis* (capítol 2 dels Resultats), es van obtenir extractes de proteïnes associades a paret cel·lular de *S. uberis* en formació de biofilm i en condicions planctòniques. A continuació, aquests extractes es van separar per electroforesi bidimensional i les proteïnes immunogèniques sobrexpressades en condicions de formació de biofilm és van detectar

diferencialment mitjançant un *pool* de sèrums de conill hiperimmuntizats amb biofilm de *S. uberis*. Per altra banda, en una segona aproximació, amb l'objectiu d'identificar proteïnes immunogèniques del biofilm expressades *in vivo*, l'extracte de proteïnes associades a paret cel·lular de *S. uberis* en formació de biofilm es va separar també per electroforesi bidimensional, i les proteïnes immunogèniques es van detectar mitjançant un sèrum d'una vaca que havia sofert una mamitis clínica per *S. uberis*. De les dues comparatives, es van identificar un total de 18 proteïnes expressades en condicions formadores de biofilm. Malgrat haver dut a terme la comparativa en extractes de paret cel·lular de *S. uberis*, es va observar que la majoria de les proteïnes identificades tenien funcions típicament associades a una localització citosòlica. Una possible explicació per aquest fet, es que la major part d'aquestes proteïnes formen part d'un grup de proteïnes conegudes com a *moonlight proteins*, que es defineixen com aquelles proteïnes que tenen més d'una funció i que es poden trobar en diferents localitzacions cel·lulars (Hernández et al., 2013; Jeffery, 2014). Així doncs, a l'hora d'escollir les proteïnes candidates a ser estudiades com a antígens vacunals, es va tenir en compte, a part de la seva funció, si havien estat descrites prèviament com a factors de virulència i/o s'havien detectat associades a la paret cel·lular en altres estudis. De la primera comparativa es va escollir la fructosa bisfosfat aldolasa (FBA), ja que havia estat descrita prèviament en *S. uberis* com una proteïna sobreexpressada en condicions formadores de biofilm respecte condicions planctòniques (Crowley et al., 2011), i localitzada també a paret cel·lular de *Streptococcus suis* (Wang et al.,

2012), *Streptococcus pneumoniae* (Ling et al., 2004) i *Streptococcus pyogenes* (Cole et al., 2005). De la segona comparativa es va seleccionar la proteïna gliceraldehid-3-fosfat deshidrogenasa (GAPDH), que havia estat prèviament descrita com un factor de virulència (Seidler, 2013) i assajada com a antigen vacunal per Fontaine i col·laboradors (2002) en un model experimental contra *S. uberis*; i finalment, el factor d'elongació Ts (EF-Ts), descrita en *Streptococcus suis* (Wang et al., 2012) i *Streptococcus pyogenes* (Cole et al., 2005) com a proteïna immunogènica associada a la paret cel·lular. A part del que es va trobar descrit a la literatura, en aquest treball es va verificar la localització d'aquestes proteïnes a la paret cel·lular de *S. uberis* mitjançant western blot. Amb aquest objectiu, es van obtenir extractes proteics de paret cel·lular i citosol de *S. uberis* i es va confirmar, amb anti-sèrums específics, que les 3 proteïnes escollides es localitzaven específicament tant a la paret cel·lular com al citosol de *S. uberis.* A continuació, es va comprovar si aquestes 3 proteïnes eren capaces de generar una resposta immunològica que protegís en front a una infecció amb *S. uberis*. Amb aquesta finalitat, es van produir de manera recombinant les tres proteïnes en *Escherichia coli*, es van formular vacunes experimentals i es va testar la seva eficàcia en un model experimental en ratolí. En aquest estudi es va observar que, malgrat totes 3 proteïnes eren capaces d'induir una resposta serològica en els ratolins vacunats, només la vacunació amb dues d'elles, FBA i EF-Ts, va aconseguir reduir significativament la mortalitat dels ratolins després d'una d'infecció intraperitoneal amb una soca virulenta de *S. uberis.* Finalment doncs, com a últim pas, es va decidir incloure totes dues

proteïnes recombinants, FBA i EF-Ts, en una formulació experimental per comprovar la seva eficàcia en un model en vaques en lactació. Malauradament, el resultats (no inclosos en aquesta tesi doctoral) van concloure que, malgrat la vacunació sí que va generar anticossos en front a les dues proteïnes, les vacunes experimentals formulades no van protegir davant una IMI amb la soca SH2U de *S. uberis*.

Paral·lelament, durant el transcurs d'aquest treball, també es va obtenir i caracteritzar un altre antigen associat a la part no proteica del biofilm de *S. uberis* (capítol 3 dels Resultats). El mètode d'obtenció d'aquest antigen es va desenvolupar en base a una metodologia d'extracció descrita prèviament a la literatura per *Staphylococcus aureus*. En la caracterització d'aquest extracte, es va identificar l'LTA mitjançant un anticòs monoclonal específic. L'LTA, és una molècula ancorada a la membrana plasmàtica dels microorganismes Gram positius, que en diverses ocasions ja ha estat descrit com un possible factor de virulència. En el cas de *S. aureus* i *E. faecalis*, s'ha demostrat que l'LTA té relació amb l'adhesió i amb la formació del biofilm, ja que la seva modificació produeix soques amb una capacitat disminuïda per formar biofilm i adherir-se a les cèl·lules MEC (Fabretti et al., 2006; Gross, Cramton, Götz, i Peschel, 2001). Específicament per *S. uberis*, un estudi d'Almeida i els seus col·laboradors (1996) ja va suggerir la relació de l'LTA i la proteïna M en l'adhesió a les cèl·lules MEC. Per altra banda, en el capítol 3 de la present tesi doctoral, es va observar una inhibició de la formació de biofilm de *S. uberis in vitro,* quan les cèl·lules bacterianes s'incubaven amb un anticòs monoclonal contra LTA, suggerint

també que aquest podria tenir un paper en la formació de biofilm de *S. uberis*. Per tant, tenint en compte la rellevància de l'LTA com a possible factor de virulència, es va decidir estudiar la seva eficàcia com a candidat vacunal. Per aquesta finalitat es va considerar adequat utilitzar un model experimental en conill. Es van formular vacunes experimentals amb l'extracte no proteic del biofilm de *S. uberis* que contenia LTA, i es va observar que, a part d'induir una resposta serològica en conilles, la vacunació va aconseguir reduir la mamitis clínica en conilles lactants després d'una infecció experimental intramamària amb una soca virulenta de *S. uberis* (resultats no mostrats en aquesta tesi). En vista dels bons resultats obtinguts, es va decidir testar l'eficàcia d'aquest candidat vacunal en vaques en lactació, tal i com es descriu en el capítol 3 de la present tesi doctoral. Amb aquest objectiu, es van vacunar dos grups de vaques gestants. El primer grup, amb dues dosis de la vacuna experimental i el segon grup amb dues dosis de PBS. Catorze dies després del part, totes les vaques van ser infectades experimentalment amb la soca SH2U seleccionada en el capítol 1 d'aquesta tesi doctoral. A continuació, es van monitoritzar durant 21 dies els signes clínics de mamitis, el RCS i el recompte bacteriològic dels quarterons infectats, així com la temperatura rectal i la producció de llet de totes les vaques. Els resultats obtinguts van demostrar que, malgrat tots els quarterons infectats van desenvolupar manifestacions clíniques de la patologia, les vaques vacunades van presentar menys signes clínics de mamitis i un menor recompte bacteriològic en els quarterons infectats. A més, després de la infecció experimental, les vaques vacunades van exhibir menor temperatura rectal i menors pèrdues en la producció de llet que

les vaques control. Finalment, les vaques vacunades van presentar un major percentatge de quarterons sans al final de l'estudi, definits com quarterons sense recompte bacteriològic i amb RCS < 200.000 cels/ml. Així doncs, els excel·lents resultats d'aquesta prova, van ser prometedors en el marc d'aconseguir una vacuna contra la mamitis bovina causada per *S. uberis*. No obstant, la limitació principal d'aquest estudi va ser no aconseguir demostrar una reducció en la incidència de casos de mamitis clínica en el grup vacunat. Una possible explicació per aquest fet és inherent als models d'infecció intramamaris, ja que en administrar-se l'inòcul per canulació de la glàndula mamària, és molt difícil evitar la replicació del microorganisme quan aquest ja és a l'interior de la cisterna mamària.

Tanmateix, amb aquests bons resultats d'eficàcia aconseguits en el treball realitzat en aquesta tesi doctoral, el desenvolupament de la vacuna experimental descrita en el capítol 3 va seguir endavant. El procés de producció de l'extracte de LTA de *S. uberis* inclòs en la vacuna estudiada al capítol 3, va ser objecte d'una sol·licitud de patent internacional PC, presentada el 14 de Febrer del 2017 i posteriorment publicada el 24 d'Agost de 2017, amb el número WO2017/140683 i títol "*Streptococuccus uberis* extract as an immunogenic agent". Per altra banda, també cal destacar que, a part de la fracció antigènica, les vacunes inactivades presenten un altre component actiu en la seva fórmula, l'adjuvant, amb la funció de potenciar la resposta immunològica contra l'antigen. Encara que els resultats no s'han mostrat en aquesta tesi, l'adjuvant de la vacuna experimental assajada en el capítol 3 (una emulsió oliosa amb monofosforil lípid A com a immunoestimulador) va ser seleccionat a

partir de diversos estudis de resposta immunològica en vedells, en els quals els animals es vacunaven amb formulacions experimentals que contenien diferents sistemes de presentació d'antigen i immunoestimuladors.

Un cop finalitzades les fases 1 i 2 del desenvolupament de la vacuna, el projecte va entrar en fase 3, on es van realitzar les proves d'eficàcia clínica de la vacuna en granges de llet, i on sí que es va poder demostrar que la vacuna reduïa la incidència de mamitis clíniques en els animals vacunats (Puig et al., 2018). Finalment, amb aquests excel·lents resultats, el projecte va entrar a la fase de registre (fase 4) i el 25 de juny del 2018, Hipra va obtenir la llicència de comercialització d'UBAC®, la primera vacuna en front a la mamitis bovina causada per *S. uberis*, a tota la Unió Europea.

Conclusions generals

5. Conclusions generals

1. Els resultats de la infecció experimental intramamària amb les soques 0140J i SU2H de *S. uberis* en vaques en lactació, suggereixen que la diferent habilitat de formació de biofilm de les soques pot conferir una estratègia de colonització i persistència diferent a la glàndula mamària.

2. La via d'administració intramamària per canulació del mugró fins a la cisterna de la glàndula mamària, amb una dosi infectiva de ≈ 100 CFU/ml de la soca SU2H, és un mètode eficaç per a reproduir la mamitis clínica en vaques en lactació.

3. La co-localització de FBA, GAPDH i EF-Ts en la paret cel·lular i el citosol de *S. uberis*, suggereix que aquestes proteïnes podrien pertànyer al grup de les *moonlight proteins*, que són aquelles proteïnes que se'ls associa una segona funció diferent a la seva principal, en alguns casos relacionada amb virulència.

4. L'LTA de *S. uberis* està implicat en la formació de biofilm *in vitro,* ja que en presència d'un anticòs monoclonal específic contra l'LTA, la seva capacitat de formar biofilm *in vitro* es veu significativament reduïda.

5. La vacunació de vaques gestants amb dues dosis d'una vacuna formulada amb l'extracte soluble de *S. uberis* que conté l'LTA obtingut en el Capítol 3 de la present tesi doctoral, redueix

la severitat de la mamitis clínica i accelera la curació dels quarterons infectats experimentalment amb una soca heteròloga i virulenta de *S. uberis.*

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