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présentée par :

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IDENTIFICATION DE BACTERIES LACTIQUES ISOLEES DE L'ECOSYSTEME MAMMAIRE BOVIN ET CARACTERISATION DE LEUR POTENTIEL INHIBITEUR CONTRE DES PATHOGENES ASSOCIES A LA MAMMITE

soutenue le 12 mars 2015 devant la commission d'Examen

Composition du jury :	Rapporteurs	Dr. Monique Zagorec
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Identificação de bactérias láticas isoladas do ecossistema mamário bovino e caracterização de seu potencial inibidor contra patógenos associados à mastite

Tese de Doutorado apresentada ao
Programa de Pós-Graduação em Microbiologia do
Instituto de Ciências Biológicas da
Universidade federal de Minas Gerais,
como requisito parcial para obtenção do grau de
Doutor em Microbiologia

Orientadores: Jacques Robert Nicoli-UFMG
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Sumário

Dedicatória

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“É melhor ser alegre que ser triste
Alegria é a melhor coisa que existe”
Vinicius de Moraes

Resumo

A mastite apresenta um impacto econômico considerável em regiões produtoras de leite de diversos países, inclusive Brasil e França. Ela é a principal causa do uso de antibióticos em fazendas leiteiras que necessitam atender à forte demanda social para que a agricultura utilize menos insumos e ajam de forma mais respeitosa com o meio ambiente e com o bem-estar animal. Dessa forma, há uma necessidade real de se encontrar ferramentas alternativas e eficazes para o controle da mastite infecciosa bovina. Esta tese teve como objectivo a pesquisa de linhagens de bactérias lácticas (BL) com capacidade inibidora de infecção mamária bovina e que possam ser utilizadas como probióticos. Para isso, 278 (165 na França e 113 no Brasil) linhagens bacterianas foram isoladas a partir de leite recém-colhido, pele do úbere e do canal do teto de vacas leiteiras. Dez linhagens de BL não redundantes foram identificadas e caracterizadas com base nas suas propriedades de parede e de produção de compostos inibidores, antes de avaliar suas interações com agentes patógenos causadores de mastite responsável e/ou as células hospedeiras em um modelo de células epiteliais mamárias bovinas (CEMB). Duas linhagens de *Lactobacillus brevis* e uma de *Lactobacillus plantarum* mostraram uma boa capacidade de adesão em células epiteliais, o que poderia inibir a invasão por *Staphylococcus aureus*, um importante patógeno associado à mastite e responsável por estimular a produção de citocinas pró e anti-inflamatórias pelas CEMB. Outros ensaios de interação com as linhagens celulares por co-inoculação revelou que a estirpe de *Lactococcus lactis* V7 foi capaz de inibir significativamente a invasão de CEMB por *Escherichia coli* e *S. aureus*. O mecanismo exacto desta inibição não foi elucidado, mas a linhagem V7 apresenta uma elevada capacidade de co-agregação a *E. coli*. *Lactococcus lactis* V7 também foi capaz de modular a resposta imune de CEMB infectadas por *E. coli* por modificar a intensidade de expressão de genes de citoquinas inflamatórias. A partir dos resultados obtidos neste trabalho, sugere-se que cepas de *L. brevis*, *L. plantarum* e *L. lactis* V7 tem características interessantes para uma possível estratégia para prevenir ou combater a mastite. Essas linhagens de BL são, de fato, capazes de inibir o crescimento e a adesão e/ou invasão de bactérias patogênicas e de modular a resposta imune das células hospedeiras.

Resumé

Les mammites ont un impact économique considérable dans les régions productrices de lait de divers pays, dont le Brésil et la France. Elles constituent la première cause de consommation d'antibiotiques dans les élevages laitiers qui doivent répondre à une forte demande sociétale pour une agriculture consommant moins d'intrants et plus respectueuse de l'environnement et du bien-être animal. Il y a donc une réelle nécessité de trouver des outils alternatifs efficaces pour le contrôle des mammites bovines infectieuses. Ce travail de thèse a eu comme objectif la recherche de souches de bactéries lactiques (BL) ayant des capacités inhibitrices de l´infection mammaire, pouvant être utilisées comme probiotiques mammaires. Pour cela, 278 (165 en France et 113 au Brésil) souches bactériennes ont été isolées à partir de lait et de la superficie et du canal du trayon de vaches laitières et 10 souches non-redondantes de BL ont été identifiées et caractérisées en fonction de leurs propriétés de paroi et de production de composés inhibiteurs avant d´évaluer leurs interactions avec des agents pathogènes responsables de mammites et/ou avec les cellules hôtes dans un modèle de cellules épithéliales mammaires bovines (CEMb). Deux souches de *Lactobacillus brevis* et une de *Lactobacillus plantarum* ont montré une bonne capacité d'adhésion aux cellules épithéliales ce qui pourrait inhiber l´invasion par *Staphylococcus aureus*, un pathogène majeur responsable de mammites, et de stimuler la production de cytokines pro- et anti-inflammatoires par les CEMb. D´autres tests d'interaction avec des lignées cellulaires en co-inoculation ont mis en évidence que la souche *Lactococcus lactis* V7 était capable d´inhiber de manière significative l'invasion des CEMb par *Escherichia coli* et *S. aureus*. Le mécanisme exact de cette inhibition n´a pas été élucidé, mais la souche *L. lactis* V7 a montré des capacités prometteuses en termes de probiotique mammaire, notamment, la capacité de moduler la réponse immunitaire des CEMb infectées par *E. coli* en intensifiant la réponse inflammatoire. A partir des résultats obtenus au cours de ce travail, on peut suggérer que des souches de *L. brevis, L. plantarum* et *L. lactis* V7 présentent des qualités intéressantes pour une possible stratégie de prévention voire de lutte contre la mammite. Ces souches sont en effet capable d'inhiber la croissance et l'adhésion et/ou l'invasion de bactéries pathogènes et de moduler la réponse immunitaire des cellules-hôte.

Abstract

Mastitis causes huge economic losses in the dairy sector both in Brazil and France. They also are the first cause of antibiotic consomption in the dairy farms. There is thus a need for new alternatives to antibiotics to control infectious mastitis. In this thesis work, we isolated 278 (165 in France and 113 in Brazil) bacterial strains from bovine milk and teat canal, and identified 10 non-redundant lactic acid bacteria (LAB) strains that were further tested as potential mammary probiotic candidates. LAB strains were tested for their surface properties and production of inhibitory compounds and then evaluated for their interactions with *Staphylococcus aureus* and *Escherichia coli*, two major mastitis pathogens, or with bovine mammary epithelial cells (bMEC), *in vitro*. Some LAB strains (*Lactobacillus brevis* e *Lactobacillus plantarum*) presented inhibitory capacity against *S. aureus* adhesion and internalisation and were shown to stimulate the production of pro- and anti-inflammatory cytokines in bMEC. Other interaction tests with bMEC showed that *Lactococcus lactis* V7 was able to significantly inhibit bMEC invasion by *Escherichia coli* and *S. aureus*. Although the inhibitory mechanism was not elucidated, *Lactococcus lactis* V7 showed promising capacities in terms of mammary probiotic potential with, notably, the ability to modulate the immune response of *E. coli*-infected bMEC by modifying the expression of inflammatory cytokine genes. In this work, several LAB strains were identified in the milk and teat canal microbiota. We showed that *L. brevis, L. plantarum* and *Lactococcus lactis* V7 strains had interesting properties for a possible strategy to prevent or treat against mastitis.

Apresentação

Para a apresentação da presente tese, as seguintes normas foram estipuladas pelo Convênio de Co-tutela Internacional de Tese assinado entre a Agrocampus Oeste (França) e a Universidade Federal de Minas Gerais – UFMG (Brasil): *A tese será redigida em português. O formato adotado será por apresentação de "artigos científicos." Sendo assim, a seção de resultados irá apresentar todo o trabalho de tese no formato de artigos científicos escritos em inglês (publicados, submetidos ou a serem submetidos para publicação). Cada capítulo terá um resumo em francês e um em português. Um resumo expandido apresentando toda a tese (incluindo as seções de introdução e discussão-perspectiva) será escrito em francês. Finalmente, resumos da tese serão escritos em português, francês e inglês.* Portanto, essa tese foi organizada em dois capítulos que abordam a busca por soluções probióticas para controle de mastite bovina. Esses dois capítulos são precedidos por uma revisão de literatura e seguidos por uma conclusão geral e perspectivas.

O primeiro capítulo trata da seleção de linhagens bacterianas láticas com potencial para uso como probiótico mamário. Para tal, linhagens de bactérias ácido láticas previamente isoladas do canal do teto de vacas leiteiras na França, e identificadas, foram avaliadas quanto a sua interação com células mamárias bovinas MAC-T. Algumas amostras se destacaram pelo alto poder de adesão às células hospedeiras, o que poderia inibir a adesão de patógenos, comprometendo sua capacidade de infecção. Essas amostras foram então avaliadas quanto a seu perfil de imunomodulação, em modelos celulares humanos, permitindo o destaque de linhagens potencialmente antiinflamatórias. O conjunto dos dados permitiu selecionar três linhagens com potencial para uso como probiótico no controle de mastites bovinas. Esse trabalho continuou, posteriormente, com a análise genômica de cinco cepas selecionadas para investigação de genes de proteínas possivelmente relacionadas à capacidade de adesão, bem como de presença de genes de resistência a antimicrobianos e será então submetido a um jornal científico internacional.

No segundo capítulo, entre linhagens de bactérias ácido láticas previamente isoladas de leite e *swab* de úberes bovinos oriundos do Brasil, foi selecionada uma linhagem de *Lactococcus lactis* denominada V7. Essa linhagem inibiu acentuadamente a invasão de células mamárias bovinas da linhagem PS por *Escherichia coli* e *Staphylococcus aureus*, dois dos principais

patógenos envolvidos na mastite. O mecanismo pelo qual essa inibição ocorre não foi elucidado, porém observou-se a capacidade de V7 em co-agregar com *E. coli*, bem como em provocar alterações na resposta inflamatória de células PS em infecções *in vitro*. Em presença de *Lactococcus lactis* V7, células infectadas expressam maiores níveis de IL-8, IL-6 e TNF-α, enquanto a expressão de TLR2 e TLR4 foi regulada. A partir dessas observações, pode ser sugerido *L. lactis* V7 como um instrumento em potencial para uso em controle de infecções mamárias bovinas. Esse trabalho foi encaminhado na forma de artigo à revista *Beneficial Microbes* (Holanda).

Présentation

Pour la présentation de le thése, les normes suivantes ont été prévues dans l'accord de co-tutelle de thèse internationale signé entre l´Agrocampus Ouest (France) et l'Université Fédérale de Minas Gerais - UFMG (Brésil): *Le mémoire de thèse sera rédigé en portuguais. Le format de rédaction adopté sera une « rédaction sur articles ». En conséquence, la section Résultats présentera l'ensemble des travaux de la thèse au format d'articles scientifiques rédigés en anglais (parus, soumis ou à soumettre pour publication). Chaque chapitre de résultats fera l'objet d'un résumé en français et en portugais. Un résumé amplifié reprenant l'ensemble de la thèse (y compris les sections introduction et discussion-perspective) sera rédigé en français. Enfin, les résumés de la thèse seront rédigés en portugais, français et anglais.*

Par conséquent, cette thèse est organisée en deux chapitres qui traitent de la recherche de solutions probiotiques pour le contrôle de la mammite bovine. Ces deux chapitres sont précédés par une revue de la littérature et suivis par une conclusion générale et des perspectives.

Le premier chapitre traite de la sélection de souches bactériennes lactiques avec un potentiel d'utilisation comme probiotiques mammaires. Pour cela, des souches de bactéries lactiques préalablement isolées du canal du trayon de vaches laitières et identifiées, ont été évaluées quant à leur interaction avec des cellules mammaires bovines MAC-T. Quelques souches se sont distinguées par leur grand pouvoir d'adhésion aux cellules hôtes, ce qui pourrait inhiber l'adhésion d´agents pathogènes, compromettant leur infectivité. Ces échantillons ont ensuite été évalués pour leur profil immunomodulateur dans une modèle cellulaire humaine, ce qui a permis de mettre en valeur des souches potentiellement anti-inflammatoires. L'ensemble de ces données a permis de sélectionner trois souches ayant un potentiel pour une utilisation en tant que probiotique dans le contrôle de la mammite bovine. Ce travail s'est poursuivi après la fin de la thèse avec l'analyse génomique de cinq souches sélectionnées pour la recherche de protéines correlationées à la capacité d'adhésion, et aussi de la présence de génes de resistence à des antibiotiques. Les résultats seront soumis à publication dans une revue scientifique internationale.

Dans le deuxième chapitre, parmi des souches de bactéries lactiques isolées de lait e de frottis de trayon bovins provenant du Brésil, nous avons sélectionné une souche de *Lactococcus lactis,* V7. Cette souche a fortement inhibé l'invasion de cellules mammaires bovines de la lignée MAC-T par *Escherichia coli* et *Staphylococcus aureus,* deux des principaux agents pathogènes intervenant dans la mammite. Le mécanisme par lequel se produit cette inhibition n'est pas été élucidé, mais il a été observé que *L. lactis* V7 présentait un léger profil pro-inflammatoire lorsqu'elle était mise en contact de MECb (lignée PS) et qu'elle intensifiait la réponse inflammatoire de cellules PS infectées par *E. coli in vitro*. En présence de *L. lactis* V7, les cellules infectées ont exprimé des niveaux plus élevés d'IL-8, IL-6 et TNF-α, alors que l'expression de TLR2 et TLR4 a été différement régulée. A partir de ces observations, *L. lactis* V7 peut être envisagé comme un outil potentiel pour une utilisation dans le contrôle des infections mammaires bovines. Un manuscript contenant les résultats de cette étuda a été soumis à la revue *Beneficial Microbes* (Pays-Bas).

Introdução e justificativa

A mastite é uma doença inflamatória, geralmente causada por infecção, que afeta rebanhos leiteiros e impacta fortemente a saúde animal, a produção e a transformação de leite. Essa doença pode ser separada em dois grupos: o ambiental, associado a micro-organismos presentes no ambiente, normalmente agravada na estação de chuvas, e o contagioso, de rápida disseminação entre os animais do rebanho. Ambos os tipos de mastite podem se apresentar na forma clínica ou subclínica assintomática. A mastite infecciosa é um dos maiores problemas de saúde animal que afetam a produção de leite no mundo, levando a grandes perdas econômicas, que pode atingir proporções de grande importância em regiões produtoras de leite de diversos países, inclusive no Brasil e na França.

O custo anual da mastite causada por *Staphylococcus aureus*, *Staphylococcus uberis* e *Escherichia coli* em rebanhos leiteiros no mundo chegou a 4.896,00 € para 100 vacas (Halasa *et al.,* 2009). A mastite causada por diferentes agentes leva a perdas de US$ 133,00 por vaca por ano, em casos de mastite por microrganismos Gram-positivo, e US$211,00 em casos associados a microrganismos Gram-negativo, considerando-se o uso de antimicrobianos, descarte de leite, descarte prematuro de vacas e redução da capacidade de produção de leite por vacas acometidas (Cha *et al.*, 2011), podendo ser ainda maior se considerados apenas os casos de mastite clínica (Huijps *et al.*, 2008).

A mastite é a principal causa do uso de antibióticos em propriedades leiteiras. A antibioterapia é utilizada tanto no tratamento quanto na prevenção da mastite, e seu uso exige grande atenção ao período de carência dos diferentes medicamentos utilizados, a fim de se evitar a venda e o consumo de leite com resíduos de antibiótico. Essa exigência é uma das grandes fontes de perdas econômicas nas propriedades leiteiras. Suscetível à recente tendência de busca por produtos obtidos de produções responsáveis, a produção de leite, bem como todas as atividades agropecuárias, se vê pressionada a responder a uma forte demanda social para uma agricultura sustentável, que utilize menos insumos e que seja mais respeitosa com o meio ambiente e o bem-estar animal. Por isso, é necessidade atual e crescente a redução do consumo de antibióticos veterinários para se evitar, entre outros problemas, o aparecimento de linhagens de agentes patogênicos, transmissíveis ao homem, resistentes aos antibióticos disponíveis no mercado.

S. aureus é a causa mais freqüente de mastite clínica em rebanhos caprinos e ovinos e sua prevalência em mastite subclínica é alta em bovinos (Cardoso, 2000; Pugh, 2005; Ericson *et al.* 2009; Botrel *et al.*, 2010). Além das mastites causadas por micro-organismos Gram positivos, aquela causada por Gram negativos é de alta relevância por ocorrer em maior freqüência nos períodos iniciais da lactação, bem como por causar maior intensidade de sinais clínicos sistêmicos (Berkama *et al.*, 1998). Dos micro-organismos Gram negativos envolvidos, *E. coli* é o mais prevalente (Lehtolainen *et al*. 2003). A contaminação do úbere bovino por esses micro-organismos é de grande importância pela alta frequência com que alcançam o leite que sai da glândula já com altos índices de contaminação (Carmo, 1999). Além da facilidade com que *S. aureus* e *E. coli* atingem o leite cru, é de grande relevância o fato de o leite ser um meio rico em nutrientes e um excelente meio de cultivo para diversos micro-organismos, que nele se desenvolvem rapidamente, desde que em temperatura de estocagem inadequada (Jay, 1996).

A fim de atender à demanda atual e reduzir o uso veterinário de antibióticos, várias estratégias de controle da mastite foram desenvolvidas, algumas já disponíveis no mercado. Porém, todos têm eficácia limitada. Este é o caso da maioria das vacinas em busca da prevenção dessa doença nos rebanhos, que têm como alvo um único micro-organismo ou um grupo restrito. Sendo a mastite uma doença multifatorial, essa estratégia deve se somar às práticas de diferentes manejos a fim de se evitar a contaminação de animais pelo solo ou entre si. Da mesma forma, a mastite causada por determinados agentes patogênicos, tais como *Staphylococcus aureus*, apresenta uma taxa de cura muito limitada após o tratamento antibiótico. Portanto, há ainda uma necessidade real de se encontrar ferramentas alternativas e verdadeiramente eficazes para o controle da mastite infecciosa bovina.

As bactérias do ácido lático (BAL) são micro-organismos envolvidos nos processamentos fermentativos do leite e são provenientes dos ecossistemas microbianos do próprio leite e do úbere. Além do envolvimento tecnológico na produção de derivados fermentados do leite, essas bactérias são importantes na preservação e proteção do ambiente mamário, sendo muitas vezes consideradas probióticas (FAO/WHO, 2002). Por serem consideradas, em sua maioria, pela Organização Mundial de Saúde, como micro-organismos seguros para o consumo humano, as BAL são de grande interesse para uso no controle de doenças. É importante, portanto, conhecer melhor micro-organismos benéficos como as BAL e suas interações, tanto

com os patógenos normalmente associados à mastite, como com as células do hospedeiro bovino. O presente estudo tem este objetivo, com ênfase nas BAL e nos patogênicos *S. aureus* e *E. coli* presentes no leite de bovinos com ou sem mastite clínica.

Esta tese teve como objetivo a procura de cepas de bactérias do ácido láctico (BAL) com capacidade inibitória da infecção mamária para que elas possam ser usadas como probióticos no ambiente do úbere. Para tal, 278 cepas de BAL foram isoladas, identificadas por sequenciamento de DNA 16S e, após seleção de linhagens de interesse, dez linhagens de BAL foram caracterizadas de acordo com as suas interações com patógenos associados à mastite e/ou com o hospedeiro em modelos de cultura celular.

Na interação com linhagens de células mamárias cultivadas, quatro linhagens de BAL foram selecionadas por suas propriedades elevadas de adesão. Essa propriedade pode ser um fator de inibição de agentes patogênicos, podendo, assim, bloquear o primeiro e fundamental passo da invasão de tecidos por micro-organismos patogênicos. Duas cepas de BAL mostraram também uma capacidade de estimular a produção de citocinas pró- inflamatórias por células epiteliais mamárias bovinas (CEMB), o que poderia ser usado como um mecanismo de controle da mastite pela modulação da resposta imune do hospedeiro.

Outros testes de interação em cultura de células por co-inoculação revelaram que a cepa *Lactococcus lactis* V7 foi capaz de inibir significativamente a invasão de CEMB por *E. coli* e por *S. aureus*. O mecanismo exato desta inibição não foi elucidado, porém a alta capacidade de co-agregação da cepa *L. lactis* V7 com *E. coli* poderia ser uma explicação plausível. *L. lactis* V7 também foi capaz de modular a resposta imune de CEMB infectados por *E. coli,* modificando a intensidade de expressão de genes de citocinas inflamatórias.

A partir dos resultados obtidos neste estudo, sugere-se que cepas de BAL, em particular *L. lactis* V7, têm características interessantes para uma possível estratégia de prevenção ou de tratamento da mastite. Essas cepas são de fato capazes de inibir a adesão e/ou a invasão de bactérias patogênicas e de modular de forma positiva a resposta imune das células hospedeiras.

Introduction et justification

La mammite est une maladie inflammatoire généralement causée par une infection qui affecte les troupeaux laitiers et qui a un grand impact sur la santé animale, la production et la transformation du lait. Cette maladie peut être séparée en deux groupes, la mammite environnementale étant associée à des micro-organismes du milieu ambiant, généralement exacerbée durant la saison des pluies, et la mammite infectieuse rapidement disseminée parmi les animaux du troupeau. Les deux types de mammites peuvent être clinique ou subclinique asymptomatique. La mammite infectieuse est l'un des principaux problèmes de santé animale affectant la production de lait dans le monde, conduisant à d'énormes pertes économiques, qui peuvent atteindre des proportions très importantes dans les régions productrices de lait de nombreux pays, dont le Brésil et la France.

Le coût annuel de la mammite causée par *Staphylococcus aureus*, *Streptococcus uberis* et *Escherichia coli* dans les troupeaux laitiers dans le monde a été estimé à 4.896,00 € pour 100 vaches (Halasa et al., 2009). Les mammites causées par différents agents entraînent des dépenses liées à l'utilisation d´antimicrobiens, à la perte de lait, à l´élimination prématurée de vaches productrices et à la réduction de la capacité de production de lait par les vaches touchées (Francis, 2005). Leur coût peut être encore plus élevé si l'on considère uniquement les cas de mammite clinique (Huijps et al., 2008).

La mammite est la principale cause de l'utilisation d'antibiotiques dans les élevages laitiers. L'antibiothérapie est strictement réglementée. Elle n'est utilisée que pour le traitement des mammites et son utilisation est exclue des démarches prophylactiques. Elle nécessite une grande attention de la part des producteurs en fonction des différents médicaments utilisés pour éviter la vente et la consommation de lait contenant des résidus d'antibiotiques. Cette exigence est une des sources importantes de pertes économiques dans les élevages laitiers. Sensible à la tendance récente de recherche de produits issus de la production durable, la production de lait, ainsi que toutes les activités agricoles, se voient pressées de répondre à une forte demande sociale pour une agriculture qui utilise moins d´intrants et qui soit plus respectueuse de l'environnement et du bien-être animal. Pour cela, il y a un besoin actuel et croissant de réduire la consommation d'antibiotiques vétérinaires pour éviter, entre autres

problèmes, l'émergence de souches d'agents pathogènes transmissibles à l'homme résistantes aux antibiotiques disponibles sur le marché.

Staphylococcus aureus est la cause la plus fréquente de la mammite clinique dans les troupeaux de chèvres et de moutons et sa prévalence est élevée en mammite subclinique chez les bovins (Cardoso, 2000; Pugh, 2005; Ericson *et al* 2009; Botrel *et al*., 2010). En plus des mammites causées par des bactéries à Gram positif, celles causées par des bactéries à Gram négatif ont un impact important: elles se produisent plus fréquemment dans les premiers stades de la lactation, et induisent des signes cliniques systémiques de grande intensité (Berkama et al., 1998). Des bactéries à Gram négatif impliquées, *Escherichia coli* est la plus répandue (Lehtolainen et al., 2003). La contamination de la mamelle bovine par ces micro-organismes a un impact important sur la transformation laitière notamment par la contamination du lait qui sort de la glande infectée (Carmo, 1999). Outre la facilité avec laquelle *S. aureus* et *E. coli* atteignent le lait cru, le fait que celui-ci soit un milieu riche en nutriments et un excellent milieu de culture pour divers micro-organismes est d'une grande importance pour qu'ils s´y développent rapidement, en cas de température de stockage incorrecte (Jay, 1996).

Afin de répondre à la demande actuelle et réduire l'utilisation massive d'antibiotiques à usage vétérinaire, plusieurs stratégies de contrôle de la mammite ont été développées, dont certaines sont déjà sur le marché. Mais toutes ont une efficacité limitée. C'est le cas de la plupart des vaccins utilisés pour la prévention de cette maladie dans les troupeaux, qui ciblent un seul micro-organisme ou un groupe restreint. Comme la mammite est une maladie multifactorielle, cette stratégie devrait être appliquée en même temps que des pratiques spécifiques de traite pour éviter la contamination des animaux par le sol ou entre eux. De même, la mammite provoquée par certains pathogènes, tel que *S. aureus*, a un taux de guérison très limité après un traitement antibiotique. Donc, il y a encore un réel besoin de trouver des alternatives vraiment efficaces pour le contrôle de la mammite bovine infectieuse.

Les bactéries lactiques (BL) sont des micro-organismes largement utilisés dans les fermentations laitières Outre l'implication technologique dans la production de dérivés laitiers fermentés, les BL sont présentes au sein de divers écosystèmes (intestinal, vaginal, mais aussi mammaire) et pourraient exercer un rôle dans la protection de l'environnement de la glande mammaire. L'Organisation Mondiale de la Santé considère la plupart des BL comme des

micro-organismes sûrs pour la consommation humaine, et les BL sont d'un grand intérêt pour une utilisation dans le contrôle de certaines maladies. Il est donc important de mieux connaître les micro-organismes bénéfiques comme les BL et leurs interactions aussi bien avec les pathogènes normalement associés à la mammite comme avec les cellules de l'hôte bovin. Notre étude a eu cet objectif, en mettant l'accent sur les BL et les agents pathogènes *E. coli* et *S. aureus* présents dans le lait de vache avec ou sans mammite clinique.

Cette thèse vise à chercher des souches de BL ayant une capacité d'inhibition de l'infection mammaire de sorte qu'elles puissent être utilisées comme probiotiques dans l'environnement de la glande mammaire bovine. Pour cela, 278 souches de BL ont été isolées, identifiées par séquençage de l'ADN 16S, caractérisées en fonction de leurs interactions avec des agents pathogènes associés à la mammite et/ou avec l'hôte en modèle de culture cellulaire.

Sur la base de leur interaction avec des cellules épithéliales mammaires bovines (CEMb) en culture, des souches de BL ont été sélectionnées pour leurs propriétés d'adhésion élevées. Cette propriété peut être un facteur d´inhibiton de pathogènes, pouvant ainsi bloquer la première étape cruciale dans l'invasion des tissus par des micro-organismes pathogènes. Certaines souches de BL ont également montré une capacité de stimuler la production de cytokines pro-inflammatoires par les CEMb, ce qui pourrait être utilisé comme un mécanisme de contrôle de la mammite par modulation de la réponse immunitaire de l'hôte.

D'autres tests d'interaction en culture cellulaire par co-inoculation ont révélé que la souche *Lactococcus lactis* V7 est capable d'inhiber de manière significative l'invasion des CEMbpar *E. coli* et *S. aureus*. *Lactococcus lactis* V7 est aussi capable de moduler la réponse immunitaire de CEMb infectées par *E. coli* par modification de l'intensité de production d'IL-8 et d'expression de gènes de cytokines inflammatoires.

A partir des résultats obtenus dans cette étude, on peut suggérer des souches de BL, dont *Lactococcus lactis* V7 en particulier, ont des caractéristiques intéressantes pour une stratégie de prévention ou de traitement des mammites bovines. Ces souches sont en effet capables d'inhiber l'adhésion et/ou l'internalisation de bactéries pathogènes et de moduler positivement la réponse immunitaire des cellules hôtes.

Revisão de literatura

Revisão de literatura

1. Ecossistema mamário bovino

1.1. Anatomia

As glândulas mamárias das fêmeas de mamíferos ruminantes apresentam em seu parênquima unidades morfofuncionais denominadas adenômeros, responsáveis pela secreção láctea. Essas unidades secretoras encontram-se albergadas e suspensas pelo estroma glandular, ou sistema suspensor da glândula, composto pela fáscia superficial do tronco, as lâminas lateral e média e lamelas de sustentação, que mantêm as glândulas fixadas à parede ventral da região inguinal. O conjunto de parênquima e estroma é denominado úbere.

O úbere de ruminantes é composto por unidades glandulares distribuídas simetricamente de cada lado da linha mediana do tronco. Nas fêmeas bovinas há quatro unidades glandulares denominadas quartos mamários, sendo dois craniais, direito e esquerdo, e dois caudais. Essas unidades são completamente separadas pelas lâminas mediais, compondo unidades glandulares independentes, produzindo quantidades distintas de leite. Essa separação impossibilita a passagem de fármacos de administração local entre os quartos. Porém, a passagem para a corrente sanguínea pode ocorrer, podendo alguns fármacos chegar assim a outros quartos (Pugh, 2005).

Cada unidade secretora se apresenta na forma de lóbulos compostos por alvéolos glandulares unidos uns aos outros e cobertos internamente por epitélio isoprismático de camada única, que produzem os componentes do leite e o secretam. Entre os alvéolos há septos de tecido conjuntivo por onde passam nervos e vasos sanguíneos e são esses septos que, quando maiores em diâmetro, separam o parênquima em lóbulos. O leite secretado pelos alvéolos é drenado pelos ductos lactíferos e, ao se unirem, formam o seio lactífero em sua porção glandular. Uma prega na mucosa, denominada Roseta de Furstenberg alberga um plexo venoso de grande importância para a vascularização do úbere e divide o seio lactífero em sua porção glandular, mais calibrosa e sua porção mais estreita, ou papilar, localizada na papila mamária ou teto (Figura 1).

Cada quarto mamário das fêmeas bovinas possui uma ligação individual com o meio externo, dando- se através da porção papilar do seio lactífero, o ducto papilar, culminando no óstio papilar, que é único por papila, diferente dos equinos e humanos, em que a ligação da glândula com o meio externo é feita por diversos óstios papilares.

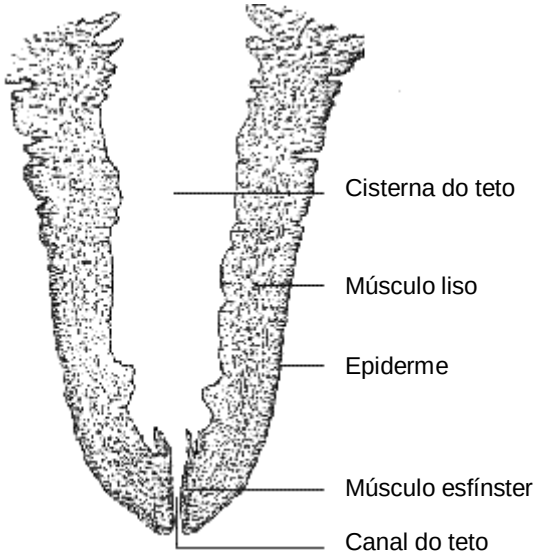


Figura 1: Glândula mamária bovina

Fonte: Unión Ganadera Regional de Jalisco
http://www.ugrj.org.mx/index.php?option=com_content&task=view&id=459&Itemid=376

1.2. Produção e secreção do Leite

A secreção do leite produzido nos alvéolos ocorre mediante estímulos táteis, visuais ou auditivos, mediados por respostas hormonais. A produção de leite nos alvéolos ocorre de forma diferenciada nos diversos tipos de mamíferos. Na glândula bovina, a secreção de leite ocorre de forma separada nas células mamárias secretórias. A gordura, as proteínas e os componentes solúveis são secretados no alvéolo e, só então se misturam, formando o leite (Foley *et al.*, 1972). Essa secreção ocorre de forma merócrina em bovinos, sem perda de material citoplasmático, o que torna a contagem eletrônica de células somáticas um bom método de monitoramento de mastite subclínica em um rebanho bovino, diferentemente da secreção na glândula caprina, que ocorre de forma apócrina, com perda parcial de material citoplasmático, o que resulta em superestimação da contagem para esses animais (Pugh, 2005).

2. Leite

2.1. Conceito

Segundo a Instrução Normativa nº 62, anexo IV (Brasil, 2011), "Entende-se por leite, sem outra especificação, o produto oriundo da ordenha completa, ininterrupta, em condições de higiene, de vacas sadias, bem alimentadas e descansadas. O leite de outras espécies deve denominar-se segundo a espécie da qual proceda". Dessa forma, o uso do termo "leite de vaca" seria uma redundância. Nesse conceito está incluído o leite cru, que por não ter sofrido processamento térmico, não está adequado ao consumo humano, visto no anexo III da mesma IN 51 (Brasil, 2002), logo não deve ser considerado um alimento, segundo RIISPOA (Brasil, 1952).

2.2. Composição do leite

Uma vez esclarecido o conceito, passamos à composição desse produto de origem animal, que varia de acordo com sua origem, bem como com a raça, a idade do animal, o número de partos, a alimentação do animal, a época do ano, o período de lactação e o momento da extração. Tomando o parâmetro legal, aceitaremos o leite bovino como referência para compreender sua composição média.

Seus componentes de maior teor percentual estão apresentados na Tabela 1.

Quadro 1: Composição quantitativa do leite

Constituinte	Limites de variação (%)	Teor percentual médio
Água	85,5 – 89,5	87,5
Sólidos totais	10,5 – 14,5	13,0
Gordura	2,5 – 6	3,9
Proteína	2,9 – 5,0	3,4
Lactose	3,6 – 5,5	4,8
Minerais	0,6 – 0,9	0,8

Fonte: Bylund, 1995

2.3. Sistemas antimicrobianos

Várias enzimas são encontradas no leite, que é uma emulsão tamponada de gordura em água. Elas atuam em diversos sistemas, em temperaturas e pH adequados. As enzimas presentes no leite têm origem biológica ou se dão por produção microbiológica e varia com a composição e tamanho da população microbiana. As enzimas naturalmente presentes no leite apresentam grande relevância na qualidade deste, por terem função de controle microbiológico, ação esta de grande importância em sua conservação nas primeiras horas, até seu completo resfriamento e processamento. Estes sistemas complexos têm duração média de três horas em leite bovino. Entre as enzimas originais de maior relevância está a peroxidase que, no leite cru, se encontra associada ao sistema lactoperoxidase, o qual utiliza, além da enzima lactoperoxidase, substratos oxidáveis como sulfidrila e tiocianato. Esse sistema tem sua função estabelecida no momento em que a enzima lactoperoxidase promove a dissociação de peróxidos naturalmente presentes no leite e, dessa reação, se desprende oxigênio. O oxigênio livre na matriz láctea oxida grupos sulfidrila (SH) de enzimas metabólicas utilizadas por micro-organismos, levando à morte de bactérias Gram negativas e à estase metabólica de Gram positivas, controlando dessa forma o crescimento bacteriano no leite cru até seu resfriamento (Jay, 1996; Franranco, 2010).

2.4. Bactérias do ácido lático

Bactérias acido láticas (BAL) são um grupo de cocos, bastonetes e cocobacilos Gram positivos composto por 12 gêneros que são *Lactococcus, Lactobacillus, Streptococcus, Leuconostoc, Pediococcus, Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus, Weissella* (Reedy *et al.*, 2008, Zhong *et al.*, 2014). Esses gêneros apresentam em comum uma baixa proporção C:G na estrutura de seu material genético e alta tolerância ao baixo pH, até porque têm, como principal produto de seu metabolismo, o ácido láctico, proveniente da via metabólica estritamente fermentativa de carboidratos. Essa produção de ácido é responsável pela redução do pH da matriz a aproximadamente quatro, o que é desejável em alimentos fermentados (Morelli, 2014; Chapot-Chartier, 2014). Além disso, são microaerofílicos ou anaeróbios facultativos. Alguns espécimes utilizam a produção de peroxidases como estratégia para sua tolerância ao oxigênio.

Esses micro-organismos heterotróficos possuem requerimentos nutricionais complexos devido a sua baixa capacidade biossintética e, por essa razão, são frequentemente encontrados associados a matrizes ricas em carboidratos, aminoácidos e vitaminas, como os alimentos derivados do leite (Pfeiler & Klaenhammer, 2007). Em meios de cultivo laboratoriais, formam colônias de aproximadamente 2 – 3 mm a 37°C. Apesar de serem, em sua maioria, mesofílicas, podem crescer em temperaturas entre cinco e 45°C (Reedy *et al*., 2008). Em virtude de suas necessidades nutricionais complexas, meios ricos devem ser utilizados para seu cultivo em laboratório (Savagodo *et al.*, 2006). Essas espécies não são capazes de formar esporos e na maior parte dos casos respondem negativamente ao teste de catalase (contudo algumas espécies podem produzir pseudo-catalase); apresentam reação de aglutinação em meios contendo hematina ou sangue e não são capazes de reduzir nitrato a nitrito, ou utilizam lactato (Carr *et al*., 2002).

Esse grupo de micro-organismos é frequentemente associado a capacidades probióticas por serem, em sua maioria, seguros para a saúde humana e animal quando consumidos (FDA, 2006). Esse *status* GRAS (*Generally Recognized As Safe*) foi conferido à maioria das espécies que compõe o grupo das BAL por serem utilizadas há muito tempo sem problemas na indústria de alimentos fermentados (FDA, 2006). Além disso, como probióticos, elas podem

possuir habilidade de aderir a células do hospedeiro, reduzindo assim a aderência de patógenos. Não são invasivas, logo apresentam menor risco de serem patogênicas, carcinogênicas, produzem substâncias antagônicas a patógenos, como peróxido de hidrogênio, ácidos e bacteriocinas e não apresentam resistência a antibióticos transmissível (Reid, 1999).

Uma estrutura de grande importância na busca pelo potencial probiótico de uma linhagem do grupo das BAL é a parede celular. Nessa, encontram-se proteínas, adesinas, capazes de aderir à mucosa do hospedeiro, refletindo na capacidade de colonizar os sítios, bem como de aderir aos patógenos ali presentes, mostrando grande importância na redução da ação patogênica de micro-organismos invasores ou oportunistas (Chapot-Chartier, 2014).

Baseado no exposto acima, a capacidade antagonista contra reveladoras patogênicas, a resistência transmissível a antibióticos e a caracterização da parede das BAL estão entre os critérios mais frequentemente avaliados durante uma seleção para uso probiótico.

2.5. *Lactococcus lactis*

Lactococcus lactis é uma BAL que se apresenta na forma de cocos Gram positivos em fileiras ou em duplas, frequentemente isolada do leite e utilizada na produção industrial e artesanal de derivados lácteos. São micro-organismos não móveis, anaeróbios facultativos e catalase negativo, que atingem maior taxa de crescimento em temperaturas próximas de 30°C, podendo crescer a 10°C. Seu metabolismo fermentativo produz ácido láctico, porém não forma gás, o que faz dessa espécie um potencial auxiliador em processos industriais (Holt *et al.*, 1993).

Seu uso industrial é consolidado na forma de culturas liofilizadas para produção de leites fermentados, manteiga e queijos, com o intuito, sobretudo, de acidificação do meio através da produção de ácido láctico. Este micro-organismo é também encontrado em leite fresco, além de derivados lácteos produzidos a partir de leite sem tratamento térmico prévio.

Apesar de a presença desse micro-organismo estar intimamente ligada aos derivados do leite, sua utilização ultrapassa tais barreiras, sendo associado à modulação imunológica (Marinho *et al.*, 2010; Zhang *et al.*, 2010) e à tecnologia de alimentos funcionais (Mercade *et al.*, 2000).

3. Mastite

Mastite é o termo que denomina condição inflamatória da glândula mamária ou, de forma geral, qualquer condição que leve a quadros de inflamação do úbere. A causa mais comum da mastite é a entrada de micro-organismos exógenos pelo canal do teto, atingindo o parênquima glandular. A entrada desses micro-organismos, porém, pode ocorrer de forma secundária, tendo um trauma ou uma ferida como porta de entrada. O desequilíbrio da condição imunológica do animal é um importante fator de risco para a instalação de agentes da mastite, que pode ser alterado por diversos outros fatores, como estresse nutricional, clima úmido ou frio, que reduz o fluxo sanguíneo à glândula ou trauma. Dessa forma, qualquer anomalia, como tetos supranumerários ou úberes baixos, que possa vir a provocar um trauma em decorrência da ordenha mecânica, trato diário ou o próprio movimento do animal, pode favorecer a ocorrência de mastite. A maior pressão produtiva imposta aos animais por sistemas de criação intensivos, por trazer maior aporte nutricional e consequente maior produção de leite, também é um importante fator que eleva a susceptibilidade ao desenvolvimento da mastite, bem como o excesso de manuseio. Em rebanhos bovinos leiteiros, a ocorrência da mastite é de 20% (Pugh, 2005). Outro fator de extrema importância para o surgimento de novas infecções de úbere é o estágio fisiológico da glândula em decorrência da fase gestacional ou de lactação do animal. O início do período seco, que coincide com o final da lactação é uma fase de transição em que cessam a limpeza e o esvaziamento frequente da glândula, proporcionando o acúmulo de leite, que é um excelente meio de cultura para micro-organismos (Botrel *et al.*, 2010) e que também leva à distensão das células secretórias, que ficam submetidas a estresse mecânico, com prejuízos à capacidade de defesa imunológica.

A mastite pode se apresentar na sua forma clínica, com sintomas aparentes ou sub-clínica, quando a única forma de monitorá-la é a contagem sistemática de células somáticas. Os sintomas observados na mastite clínica são aumento da temperatura externa do úbere, tumefação, presença de dor e rubor e, quando os quartos posteriores forem afetados, claudicação ou andar rígido (Pugh, 2005). A visualização desse tipo de mastite é facilitada pela presença de grumos nos primeiros jatos de leite à ocasião da ordenha. Casos de mastite clínica causados por *Escherichia coli* ou *Mycoplasma* spp., não tratados adequadamente, podem evoluir para septicemia, evidenciada pelo aumento da temperatura retal, da frequência

cardíaca, inapetência e redução acentuada e repentina da produção de leite, podendo levar ao óbito. A mastite clínica pode evoluir ainda para o quadro gangrenoso, sobretudo em ovinos, em virtude da redução do fluxo sanguíneo para a glândula após a formação do edema. O micro-organismo mais frequentemente associado a tal quadro é *Staphylococcus aureus* (Nader Filho, 2007) e a prevalência dessa infecção aumenta com a idade do animal e o número de lactações. As evidências dessa infecção são a presença de edema e eritema iniciais no úbere, que sofre posterior queda de temperatura e desenvolve coloração cianótica em decorrência da isquemia. Em estágio mais evoluído do quadro, há presença de sinais de infecção sistêmica, como aumento de temperatura retal, inapetência, recusa em se deitar e claudicação em razão de dor glandular. A mastite bacteriana pode levar a um quadro clínico que pode ser tratado com antibióticos, porém o prognóstico é desfavorável. Nesses casos há formação de abscessos no úbere, o que torna os animais infectados fontes de contaminação para os animais sadios e leva à manutenção de mastite sub-clínica no rebanho. Essa transmissão ocorre por práticas errôneas de higiene do equipamento de ordenha ou do próprio pessoal envolvido na ordenha. Os micro-organismos mais frequentemente associados a essa enfermidade em bovinos são *Staphylococcus aureus* (Cardoso *et al.*, 2000) em mastites clínicas, e *Staphylococcus* coagulase negativo e *Streptococcus dysgalactiae* em mastites sub-clínicas (Botrel *et al.*, 2010), tendo ainda como terceiro agente mais importante *Escherichia coli* (Ericson *et al.*, 2009). Sabe-se, porém, que a detecção desses micro-organismos é subestimada por baixa sensibilidade dos métodos utilizados (Viora *et al.*, 2014).

Os diversos tipos de mastite são abordados atualmente com o uso intenso de antimicrobianos, apesar de o controle não ser efetivo. Em casos clínicos ou agudos, trata-se o animal com antimicrobiano intra-mamário e se interrompe a coleta de leite pelo período de carência indicado pelo fabricante do medicamento utilizado, a fim de evitar a presença de resíduos de drogas no leite. Esse método leva a grandes perdas econômicas devido ao descarte desse leite, à injúria da glândula do animal, com possíveis consequências para as lactações futuras e necessidade de descarte prematuro de animais em idade produtiva. Uma alternativa atualmente utilizada é o tratamento de todos os animais em idade de lactação com antimicrobianos intra-mamários na entrada do período seco, ou entre lactações, que é normalmente de dois meses. Esse procedimento evita o surgimento de casos clínicos. Algumas propriedades fazem o monitoramento da presença de animais com mastite sub-clínica pela contagem de células somáticas (CCS), que mede o nível de reação inflamatória no úbere. A presença de contagens acima de $7,5 \times 10^5$ células por mL (Brasil, 2011), é

considerada como presença de mastite sub-clínica. Faz-se um antibiograma para esses animais, que são, então, tratados no período seco com o antimicrobiano específico. O gasto com esse procedimento é de aproximadamente US$110,00 por vaca em lactação por ano, nos Estados Unidos (Francis, 2005) ou ainda US $325,00 por vaca com mastite clínica (Huijps *et al.*, 2008). A frequente não obediência do período de carência da droga leva à recepção de leite com resíduos não detectáveis de antimicrobianos pelos métodos rápidos, causando um problema industrial de inibição do fermento lático utilizado na produção de leites fermentados e queijos, bem como de saúde pública, representado pelo risco de seleção de linhagens resistentes ao tratamento com antimicrobianos. Em quadros de mastite bovina, foram observadas linhagens de *Staphylococcus* spp. resistentes à penicilina G e de *Streptococcus* spp. resistentes a macrolídios e tetraciclinas (Botrel *et al.*, 2010), além de outras drogas (Fessler, 2010). Apesar de a taxa de resistência encontrada ter sido inferior à encontrada em linhagens isoladas de humanos, salienta-se o risco de sua transferência pelo consumo de leite cru ou pela manipulação na ordenha.

Staphylococcus aureus é o principal agente causador de mastite em bovinos (Cardoso *et al.*, 2000; Pugh, 2005) e algumas linhagens desse micro-organismo são capazes de produzir grandes quantidades de toxinas extracelulares e de fatores de virulência (Fijalkowski, 2014). A presença dessas toxinas no leite ou outro alimento derivado lácteo pode gerar graves consequências para a saúde humana e seu controle se torna uma necessidade de grande importância para a saúde pública (Baumgartner, 2014). Como alternativa ao uso de antimicrobianos para controle da mastite bacteriana, sugere-se o uso de micro-organismos probióticos, assim como para um possível controle de mastite sub-clínica, evitando o surgimento de casos clínicos que deveriam ser tratados com antimicrobianos.

3.1 *Staphylococcus aureus*

As bactérias do gênero *Staphylococcus* possuem características genéticas, fisiológicas e bioquímicas diversas e, morfologicamente, *Staphylococcus* spp. são caracterizados como cocos Gram positivos imóveis, não esporulados, que se agrupam em grupo irregular. Essa formação, semelhante a cachos de uva, foi responsável pela denominação do gênero, sendo *staphyle*, cachos de uva em grego, e *coccus*, grãos (Baird-Parker, 1990). Bioquimicamente, cada espécie se comporta de maneira diferente, podendo ser positiva ou negativa para as

provas de coagulase, catalase e termonuclease. São anaeróbios facultativos, medindo cerca de 0,5 a 1 µm de diâmetro (Baird-Parker, 1974; Harrys *et al*., 2002).

Esses micro-organismos são habitantes naturais de pele e mucosa de mamíferos, mãos, leito sub-ungueal, fossas nasais e orofaringe de humanos saudáveis. Estão, ainda, frequentemente envolvidos em inflamações intra-mamárias de fêmeas em lactação, sendo o principal agente causador da mastite em bovinos (Nader Filho *et al.*, 2007).

Frequentemente envolvido em quadros de mastite bovina, essa espécie representa grande risco de contágio no sistema de produção, afetando normalmente grande parte do rebanho ou todos os animais em lactação, levando a enormes prejuízos. Esse gênero adquire grande importância dentro do grupo por apresentar a capacidade de produzir toxinas que, ao serem ingeridas, provocam quadros graves de intoxicação alimentar em animais e humanos. Algumas linhagens de *Staphylococcus aureus* produtoras do superantígenos TSST1, ou toxina do choque tóxico, levam a quadro clínico extremamente grave de mastite, podendo provocar a perda do quarto da glândula afetado, com proporcionais perdas econômicas.

Sendo a mastite uma enfermidade frequente no rebanho brasileiro e *Staphylococcus* spp. um micro-organismo frequentemente envolvido, compreende-se que boa parte do leite cru produzido no país apresenta certos níveis de contaminação, principalmente de *Staphylococcus aureus*, apesar de *Staphylococcus intermedius* também estar presente em produtos derivados de leite (Sena, 2000; Nader Filho *et al.*, 2007).

Os queijos produzidos com leite cru são facilmente atingidos por contaminações com *Staphylococcus* spp. em virtude de não passarem por processamento térmico. As contaminações podem atingir contagens altas (Le Loir *et al.*, 2003; Charlier *et al.*, 2009) em queijo de coalho, com 10^6 UFC/g de *Staphylococcus aureus* produtor de enterotoxinas, principalmente do tipo B (SEB). Vistos tais níveis de contaminação e a facilidade com que pode atingir o consumidor final, esse estudo se faz necessário para avaliar uma ferramenta de controle alternativa (probióticos) dessa contaminação nos rebanhos leiteiros.

3.2. *Escherichia coli*

As bactérias do gênero *Escherichia* pertencem à família *Enterobacteriaceae* e se apresentam em forma de bastonetes Gram negativos não esporulados, não móveis e anaeróbios facultativos. Estão normalmente presentes no conteúdo gastrointestinal de mamíferos, inclusive dos bovinos, em uma relação simbiôntica. Porém, diversas linhagens da espécie *Escherichia coli* adquirem a capacidade de causar quadros infecciosos em seu hospedeiro. O seu controle nos ambientes de descanso dos animais se torna, portanto, a linha de frente no combate às mastites ambientais nos rebanhos leiteiros. Contudo, durante a estação de chuvas, em que a temperatura é também favorável para o desenvolvimento desse micro-organismo no ambiente, esse controle se torna limitado, sendo necessário manejo eficiente e limpeza rigorosa dos estábulos.

De importância capital na prevalência e gravidades de quadros de mastite bovina ambiental, *Escherichia coli* é um patógeno de difícil controle (Suojala *et al*., 2013). Quando envolvidos em contaminações da glândula mamária bovina, esse micro-organismo leva frequentemente a quadros agudos e sistêmicos, com perda imediata na produção de leite do animal. Tendo em vista o difícil controle com manejo, o uso de antimicrobianos durante a época das chuvas é aumentado.

4. Probióticos

O termo "probiótico" foi definido, inicialmente, como "organismos vivos que, quando ingeridos, exercem efeito benéfico no balanço da microbiota intestinal do hospedeiro" (Fuller, 1989). Esse conceito foi, posteriormente, modificado para "organismos vivos que, quando administrados em quantidade adequada, exercem efeitos benéficos para a saúde do hospedeiro" (FAO/WHO, 2002).

As culturas de micro-organismos probióticos usadas para prevenção de infecções devem produzir substâncias de interesse, de acordo com o efeito desejado, ou promover sua ação benéfica por outra via. Os micro-organismos probióticos, dependendo da natureza da cultura, têm potencial de conferir diferentes efeitos benéficos para a saúde humana, se administrados de forma regular, pois até então não foi comprovada sua instalação definitiva nos sítios de

aplicação. Para que seja viável o uso desses micro-organismos, eles devem resistir às etapas do processamento industrial do produto, ter velocidade de crescimento adequada a sua produção, resistir às condições do órgão ou tecido a que se destina, serem reconhecidamente seguros para a saúde humana, GRAS *Generally Recognized As Safe* (Reid, 1999) e, obviamente, apresentar algum benefício para o hospedeiro no qual é administrado.

Objetivos

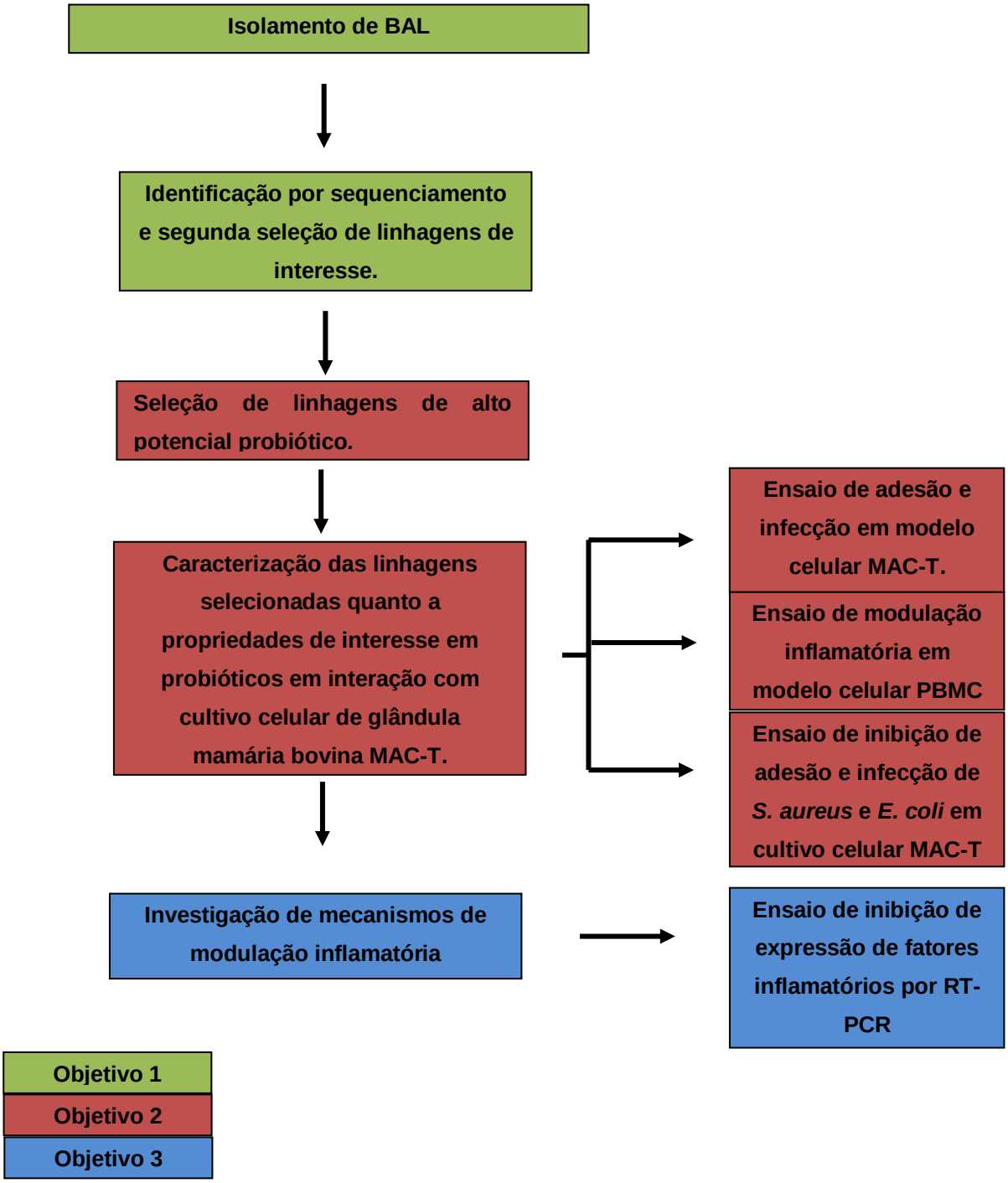
1. Objetivo geral

Isolar, identificar e caracterizar linhagens de BAL do ecossistema mamário bovino quanto a seu potencial probiótico para tratamento ou prevenção da mastite.

2. Objetivos específicos

1. Isolar e identificar linhagens de BAL no ecossistema mamário bovino;
2. Selecionar, entre as linhagens de BAL isoladas, as candidatas ao uso probiótico, avaliando as capacidades de adesão, internalização e imunomodulação em cultura de células.
3. Avaliar as capacidades de imunomodulação e de inibição da adesão e invasão patogênica num modelo de co-inoculação em cultura de células de BAL com *Staphylococcus aureus* ou *Escherichia coli*.

Estratégia de trabalho



Capítulo I

Resumo

A mastite bovina é uma doença veterinária que causa grandes perdas econômicas em diversos países. A prevenção e o tratamento dessa doença são baseados na antibioticoterapia, o que, além de levar a perdas industriais, é um problema para a saúde pública. As desvantagens relacionadas à terapia baseada no uso de antimicrobianos são somadas ao fato de não apresentar boa eficácia, sobretudo contra *Staphylococcus aureus*, um dos mais relevantes patógenos associados à mastite. Nos últimos anos, a utilização de microrganismos probióticos na prevenção de infecções em humanos ganhou interesse, após uso já consolidado em animais. O uso de probióticos contra infecções se iniciou no trato gastrointestinal, porém mais recentemente, esse uso se expandiu para outros ambientes, como o vaginal e o cutâneo. O objetivo desse estudo foi isolar, do ecossistema mamário bovino, bactérias ácido-láticas (BAL) que apresentem propriedades benéficas que possam ser utilizadas para a prevenção e/ou o tratamento de mastites bovinas, além de serem seguras para uso animal.

A amostragem a partir do ducto do teto, prévia ao presente trabalho, permitiu o isolamento de 165 linhagens pertencentes aos gêneros *Enterococcus, Streptococcus, Lactobacillus* e *Lactococcus*. Dez linhagens geneticamente não redundantes – determinadas por eletroforese em campo pulsado (PFGE) – correspondendo a espécies não patogênicas foram selecionadas com base em suas propriedades de superfície celular (hidrofobicidade e auto-agregação), além de capacidade de produção de compostos antimicrobianos.

Como parte do presente trabalho de tese, as dez cepas selecionadas anteriormente tiveram seu perfil de adesão e internalização à linhagem de células mamárias MAC-T avaliado. Foi ainda avaliada sua capacidade de imunomodulação da mesma linhagem celular bovina. Das dez cepas avaliadas foram selecionadas cinco com base em sua capacidade de adesão celular, bem como de suas propriedades imunomodulatórias.

Posteriormente ao trabalho desenvolvido como parte do presente doutorado, as cinco cepas selecionadas tiveram seu genoma sequenciado. Essa análise permitiu identificar a presença de fatores de risco ao uso como probiótico, tal como resistência a antibióticos, permitindo uma avaliação mais crítica e realista sobre a possibilidade do uso dessas cepas em terapias contra a

mastite bovina. Essa análise permitiu, ainda, identificar alguns fatores determinantes de algumas propriedades probióticas, tais como adesão e auto-agregação.

A análise dos resultados obtidos nesse estudo permitiu a seleção de linhagens de BAL do ecossistema mamário bovino que apresentaram propriedades benéficas para a prevenção ou tratamento de mastites bovinas. Além disso, foi possível identificar genes que codificam proteínas relacionadas à capacidade de adesão celular.

Resumé

La mammite bovine est une maladie vétérinaire qui provoque des pertes économiques majeures dans plusieurs pays. La prévention et le traitement de cette maladie sont basés sur l'utilisation d'antibiotiques qui ne montrent pas une bonne efficacité, en particulier contre *Staphylococcus aureus*. En outre les antibiotiques doivent être éliminés des animaux traités ce qui entraine un manque à gagner pour les éleveurs et retarde la transformation laitière. Enfin, leur utilisation massive entraine un risque d'émergence de résistances et constitue potentiellement un probléme de santé publique. Au cours des dernières années, l'utilisation de microorganismes probiotiques pour la prévention des infections chez l'homme a acquis un intérêt croissant, après une utilisation consolidée chez les animaux. L'utilisation des probiotiques pour les infections a d´abord commencé pour des applications visant l'équilibre microbien du tractus gastro-intestinal, et plus récemment, ces applications se sont étendues à d'autres environnements, comme les microbiotes vaginaux et cutanés. Le but de cette étude a donc été d'isoler de l'écosystème mammaire bovin, des bactéries lactiques (BL) ayant des propriétés bénéfiques qui puissent être utilisées pour la prévention ou le traitement de la mammite bovine.

L'échantillonnage du canal du trayon a permis l'isolement de 165 souches appartenant aux genres *Enterococcus, Streptococcus, Lactobacillus* et *Lactococcus.* Dix souches génétiquement non redondantes - déterminé par électrophorèse en champ pulsé (PFGE) - correspondant à des espèces non pathogènes ont été sélectionnées sur la base de leurs propriétés de surface cellulaire (d'hydrophobicité et d'autoagrégation), de capacité de production de composés antimicrobiens, de capacités d'adhésion ou d'internalisation cellulaire et de propriétés immunomodulatrices.

Des dix souches caractérisées, cinq ont eu leur génome séquencé. Cette analyse a identifié la présence de facteurs de risque à l'usage comme probiotique, tels que la résistance aux antibiotiques, ce qui permet une évaluation plus critique et réaliste quant à l'utilisation possible de ces souches dans des thérapies contre la mammite bovine. Cette analyse a également permis d'identifier quelques déterminants de certaines propriétés probiotiques telles que l'adhérence et l´autoagregation.

L’analyse des résultats obtenus dans cette étude a permis la sélection de souches de BL de l’écosystème mammaire bovin, qui ont des propriétés bénéfiques pour la prévention ou le traitement de la mammite bovine. En outre, il a été possible d'identifier des protéines liées à la capacité d'adhérence cellulaire.

Probiotic properties of lactic acid bacteria from mammary microbiota

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Abstract

Bovine mastitis is a costly disease in dairy cattle worldwide. Prevention and treatment of this inflammation of the mammary gland, mainly based on vaccines and antibiotics are not fully efficient, thus prompting the need for alternative strategies. The goal of this study was to isolate autochtonous lactic acid bacteria (LAB) from the bovine mammary microbiota exhibiting beneficial properties that could be used for mastitis prevention or treatment. Sampling of the teat canal led to the isolation of 165 isolates, among which a selection of 10 non-redundant LAB strains –as determined by PFGE- were further characterized, with regard to several properties: surface properties (hydrophobicity, autoaggregation); inhibition potential of the three main mastitis etiological agents, i.e. *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus uberis;* colonization capacities of bovine mammary epithelial cells and immunomodulation properties. Three strains, namely *Lactobacillus brevis* 1595 and 1597 and *Lactobacillus plantarum* 1610, were characterized by high colonization capacities and a medium surface hydrophobicity. These strains are good candidates to compete with pathogens for mammary gland colonization. Besides, *Lactobacillus casei* 1542 exhibited a pro-inflammatory profile (IL8 production) which could be useful to stimulate the bovine mammary innate immune system. Full genome sequencing of five of these candidate strains allowed to check for risk factors such as antibiotic resistance genes and to identify potential bacterial determinants involved in the beneficial properties. This work allowed the selection of promising candidate LAB strains from the bovine mammary microbiota that harbored beneficial properties for the prevention or treatment of bovine mastitis.

Keywords: Mammary microbiota, Lactic Acid Bacteria, mastitis, ruminants, probiotics

Introduction

Bovine mastitis is defined as an inflammation of the mammary gland and most often results from bacterial infection. These intramammary infections cause huge economic losses in the dairy farming and industry (1, 2). As of yet, the control of bovine mastitis is mostly based on antibiotics. However, they are not totally efficient and contribute to the emergence and transmission of antibiotic resistance within the host microbiota, which include both commensal and opportunistic pathogens. There is thus a need for alternative strategies, which can be used as prophylactic treatment or as an alternative or a complementary curative treatment.

One of these alternatives is the emerging concept of mammary probiotics. For this purpose, LAB are good candidates thanks to their GRAS status and to their recognized technological and inhibitory properties. LAB have been investigated for many years for their beneficial effects on the human health (3–6). They contribute to maintain the balance of natural microbiota (i.e. vaginal and gut), by competing with pathogens for tissue colonization, modulating virulence expression or stimulating the innate immune system (7–9).

Likewise, the use of probiotics has gained interest in the veterinary community. The autochthonous bovine mammary microbiota was investigated, either by isolation on selective culture media or by 16S rRNA sequencing to identify micro-organisms with inhibitory properties against mastitis pathogens (10, 11). A bacteriocin producing *Lactococcus lactis* was shown to be as efficient, *in vivo*, as a conventional antibiotic treatment to treat cow mastitis(12–14). Encouraging results were also obtained *in vitro* with a strain of *Lactobacillus perolens*, which was shown to inhibit several mastitis causing pathogens thanks to its coaggregation with mastitis pathogens and its colonization capacities to bovine mammary epithelial cells (bMEC) (15). When used in *in vivo* intramammary injection, this strain did not show adverse effect on mammary tissue (16). Similarly, we recently demonstrated *in vitro*

that different *Lactobacillus casei* strains, including one strain isolated from bovine teat canal, inhibit adhesion and internalization of *S. aureus* to bMEC without affecting the bMEC physiology (17).

Based on these observations, the objective of this work was to isolate LAB from the bovine mammary microbiota, and to characterize their beneficial properties so as to select good candidates to be included in a mammary probiotic cocktail against infectious mastitis. As beneficial properties, we first evaluated capacities of LAB strains to inhibit growth of the three main pathogens associated to mastitis, i.e. *Staphylococcus aureus, Escherichia coli and Streptococcus uberis*, through acidification and production of hydrogen peroxide and bacteriocin-like compounds (18). Secondly, we characterized their surface properties including autoaggregation capacities and degree of hydrophobicity, which have been associated to the ability to colonize the host tissues (19). Thirdly, we investigated the capacities of these LAB strains to adhere to bMEC (MAC-T cell line), so as to estimate their potential to colonize the mammary gland epithelium *in vivo* and as a consequence, to compete with pathogens for tissue colonization. Last, their ability to stimulate the innate immune system (pro- and anti-inflammatory properties) was estimated by measuring their capacity to stimulate the production of a pro-inflammatory cytokine (IL8) and an anti-inflammatory cytokine (IL10). This was done in two different cellular models, HT29 and PBMC respectively, which have been widely used to characterize immunomodulation potential of LAB strains (20). The full sequencing of 5 out of 10 strains was included so as to identify potential genomic determinants of the colonization and immunomodulation capacities and to exclude any risk factors, e.g. antibiotic resistance determinants.

This characterization allowed us to identify promising LAB strains exhibiting a good potential to colonize the mammary gland ecosystem and or immunomodulation properties.

Material and methods

Sampling. The samples were collected from 20 Holstein dairy cows in two herds belonging to the InterBioBretagne network (organic farming organization), in the region of Brittany in France. One quarter per cow was sampled, corresponding to the left or right rear quarter. Teats were thoroughly washed with water and cleaned with ethanol 70 % and individual paper towels. Teat canals were then sampled in two different ways. A 5 mm sterile Histobrush® swab (D. Dutscher, Brumath, France), was inserted 5 mm inside the teat apex and turned 3 times before removal. The swabs were immediately placed in tubes containing 2 mL of sterile peptone solution (peptone 20 g/L sodium chloride 5 g/L). Foremilk samples were then collected in sterile plastic tubes. All sample were stored on ice until processing in the laboratory.

Isolation of LAB strains. Foremilk samples were homogenized with 9 volumes of a trisodium citrate solution (2 % w/v) and centrifuged (6650 g / 5 min / 4 ºC). Pellet was then resuspended in 2 mL of sterile peptone solution. Bacterial suspensions corresponding to swab and foremilk samples were enriched on M17, MRS or MRS 5.4 by diluting 100 µL of bacterial suspension in 12 mL of M17, MRS at pH 6.8 and MRS acidified at pH 5.4 (hereafter named MRS 5.4, more selective for Lactobacilli) followed by a 48 h incubation at 37 °C in anaerobic jar, for elective cultivation. Serial dilutions of the enriched bacterial suspensions were then performed, plated on M17, MRS and MRS 5.4 and incubated for 48h at 37°C in an anaerobic jar. Colonies with different morphotypes were isolated and set in collection in the enrichment medium supplemented with 15 % glycerol and stored at -80 °C.

Genetic identification of isolates. Each isolate was identified by sequencing the 16S rDNA gene. Genomic DNA was isolated from a 2 mL overnight culture on M17, MRS or MRS 5.4

after centrifugation (6000 g / 5 min / 4 ºC), washing with 1 mL of peptone solution, and an additional centrifugation (6000 g / 5 min / 4 ºC). The pellet was lysed for 45 min at 37°C in 180 µL lysis buffer containing 20 mM Tris-HCl (pH 8), 2 mM EDTA, 1% triton X100 and 20 g/L lysozyme (MP Biomedicals, Illkirch, France). Genomic DNA was purified using the kit DNeasy® Blood & Tissue Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacter's recommendations.

PCR amplification of 16S rDNA was performed using a Veriti™ 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) in a 50 µL final volume containing 20 ng genomic DNA, 1x HF Phusion buffer, 0.5 µM of primer W001 (5'-AGAGTTTGATCMTGGCTC) and W002 (5'-GNTACCTTGTTACGACTT), 200 µM dNTP and 1U Phusion polymerase (New England Biolabs). The PCR conditions were: denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min 30 s. A final extension step was performed for 10 min at 72 °C. Sequencing of the PCR product was performed by LGC Genomics (Berlin, Germany).

The 22 LAB strains retained for PFGE analysis (see results) have been registered in the collection of the CIRM-BIA Biological Ressource Center (Rennes, France).

Characterization of LAB isolates by Pulse-field gel electrophoresis. The PFGE molecular fingerprints of LAB isolates were obtained using the method adapted from Smith and Cantor (21). The culture and the agarose blocks were prepared as described previously (22). The blocks were equilibrated for one hour in a restriction buffer at 4 °C and transferred to 300 µL fresh digestion buffer containing 15 U of *Sma*I or 25 U of *AscI* endonucleases (New England Biolabs, Hitchin, United Kingdom). The blocks were incubated overnight at 25°C for *Sma*I and for 4h at 37 °C for *AcsI*. PFGE was carried out with a CHEF-DR II apparatus (Bio-Rad,

Australia) in a 1% agarose gel (w/v) (Ultrapur, Gibco-BRL, Scotland) in 0.5× TBE at 200 V and at 14 °C with following pulsed times and total running time: *Sma*I (initial time — 10 s, final time — 10 s, total running time — 24 h), *AscI* (10 s, 15 s, 22 h). After electrophoresis, gels were stained with GelRed and visualized under UV light. Photographs of PFGE gels were scanned, and the band profiles were analyzed using BioNumerics, version 4.1 (Applied Maths, Kortrijk, Belgium). Comparisons between the normalized band profiles were made using the Dice similarity coefficient. Clustering of strain profiles was accomplished by using unweighted pair group method with arithmetic averages (UPGMA) and standard deviation 3.3%.

Production of hydrogen peroxide. Screening of hydrogen peroxide production by the isolates was performed using a TMB (3,30,5,50-tetramethylbenzidine) assay, as described previously (11). Strains were classified, according to the intensity of the color, as non-producers, low producers or high producers of H_2O_2.

Production of antagonistic substances. The screening of the antimicrobial potential of the LAB supernatants was carried out using agar plate diffusion method as previously described (23). Supernatants were either native, neutralized with NaOH or neutralized and treated with 1000 U/mL of catalase (Sigma Aldrich, USA) during 1 h at 25 °C. Six pathogenic strains, corresponding to the main species involved in bovine mastitis, were used as indicators: *Staphylococcus aureus* RF122 and Newbould 305 (N305), which were isolated from bovine mastitis (24–26), *Streptococcus uberis* LMA1675 and LMA1672 and *Escherichia coli* LMA1678 and LMA1674. *S. uberis* and *E. coli* strains were isolated from the mammary ecosystem during this study.

Bacterial surface properties. Autoaggregation capacities (i.e. the capacity of a strain to form aggregates in a bacterial suspension) was determined as described previously (11). The degree of hydrophobicity was evaluated using the microbial adhesion to hydrocarbons method (MATH) with hexadecane (Sigma Aldrich, US) (27). Strains were classified as low, medium and high according to their hydrophobicity or autoaggregative capacities.

Mammary epithelial cells and culture conditions. The established bMEC MAC-T line (Nexia Biotechnologies, Quebec, Canada) was cultured in T75 cell culture flasks in DMEM containing 10 % heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 10 mg/mL streptomycin, and 5 µg/mL insulin (D. Dutscher). Cells were incubated at 37°C in a humidified incubator with 5 % CO_2. They were cultured to a confluent monolayer, treated with 0.05 % trypsin (Gibco-BRL, Grand Island, NY), and suspended in fresh MAC-T medium at a concentration of $2x10^5$ cells/mL. For adhesion and internalization assays, cells were then seeded in 12-well plates ($2x10^5$ cells/well) and incubated overnight at 37°C in 5 % CO_2 to obtain a confluent monolayer.

Adhesion assay. Adhesion assays were performed as described previously (17). Briefly, confluent monolayers of MAC-T cells ($2.5x10^5$ cells/well) were washed twice with PBS and incubated at 37°C in 5% CO2 with 1mL of LAB suspensions in DMEM at $1x10^8$ CFU/mL or $5x10^8$ CFU/mL to achieve a ratio of interaction (ROI) of LAB organisms to cells of 400:1 and 2000:1 respectively. LAB adhesion was measured 1 h post-interaction. After washing 4 times with PBS, the monolayer was treated with 0.05 % trypsin, centrifuged for 5 minutes at 800 g and lysed with 0.01% triton. The population of LAB that adhered to the cells was determined by colony counting, on M17 for lactococci and MRS for lactobacilli, from serial dilutions of the cell lysates.

Internalization assays. Internalization assays were performed in the same conditions as adhesion assays (same ROI) except that LAB internalization was measured 2 h post-interaction. Following incubation with LAB, cells were washed 4 times with PBS and incubated for 2 hours with 1.0 mL of DMEM containing 100µg/mL gentamicin in order to kill extracellular bacteria and allow the numeration of the internalized bacterial population only. Subsequently, cells were lysed and the population of internalized LAB was determined as described above.

Immunomodulatory effects of LAB strains on PBMC model. Human PBMCs were obtained from three healthy donors and isolated as previously described (28, 29). The cytokine induction pattern was evaluated as previously described (20). The LAB strains were grown twice for 16h at 37°C in M17 or MRS to reach the early stationary phase. They were washed twice in phosphate-buffered saline (PBS), and resuspended in PBS. The bacterial density was adjusted to OD_{600} =1 (corresponding to approximately $5x10^8$ CFU/mL). PBMCs ($1x10^6$ cells/mL) were seeded in 24-well tissue culture plates. Bacterial suspensions were added to obtain ROI 10:1. PBS was used as a negative (non stimulated) control. *Lactobacillus rhamnosus* GG, which is known to induce IL10 production (30, 31) was used as reference. For each donor, three wells per bacterial strain were used. After 24 h stimulation at 37°C in air with 10 % CO_2, culture supernatants were collected, mixed with anti-protease cocktail as indicated by manufacturer (complete, EDTA-free tablets, Roche) and stored at -80°C until cytokine analysis. Neither medium acidification (red phenol as pH marker) nor bacterial proliferation was observed. IL-10 concentrations were measured by enzyme-linked immunosorbent assay (ELISA) (Mabtech, Sweden) according to the manufacturer's recommendations.

Immunomodulatory effects of LAB strains on HT-29 cell line. Experiments were performed as previously described (20). Briefly, experiments were initiated when HT-29 cells were at confluence (~1.83×10^6 cells/well). LAB were added at ROI 40:1 in 50 µL DMEM in a total volume of 500 µL. Cells were stimulated simultaneously with recombinant human TNF-α (5 ng/mL; Peprotech, NJ) for 6 h at 37°C in 10 % CO_2. Stimulation of HT29 cell line with TNF-α alone was used as the reference condition. All samples were analyzed in triplicate in three independent assays (9 data points in total including 3 technical replicates per assay). After coincubation, cell supernatants were collected, mixed with anti-protease cocktail as indicated by manufacturer (Complete, EDTA-free tablets, Roche) and frozen at −80°C until further analysis of interleukin-8 (IL-8) concentrations by ELISA (Biolegend, San Diego, CA).

Genome sequencing. Genomic DNA of *L. brevis* 1595, *L. casei* 1542, *L. lactis* 1596, and *L. plantarum* 1610 and 1612 was extracted and purified as described above. Genome sequencing, assembly and annotation were performed as previously described (24). Coding sequences (CDSs) detection was performed with the Glimmer3 software application (PubMed identification no. [PMID] 17237039). Gene products were subjected to protein location prediction using the software package SurfG (32). Genomes were further screened using the CD-search tool of the Conserved Domain Database for the presence of specific domains (33, 34). In particular, all genomes were screened for the presence of domains involved in binding to mucin, collagen and fibronectin: pfam06458 (MucBP): mucine-binding protein domain; cl05785: MucBP superfamily, pfam5737 (Collagen_bind): Collagen binding domain; pfam05738 (Cna-B): Cna protein B-type domain (this domain is found in *Staphylococcus aureus* collagen-binding surface protein. However, this region does not mediate collagen binding); cl15753 (collagenBindB superfamily); cl05349 (collagen_bind superfamily); pfam07299 (FBP): fibronectin-binding domain; pfam05833 (FbpA): Fibronectin-binding

protein A N-terminus; cl06363: FBP superfamily; pfam00497 (SBP-bac-3): bacterial extracellular solute binding proteins, family 3. Systematic analysis of conserved domain content of proteins annotated as Internalin was also included.

In addition, all genomes were screened for the presence of genes potentially encoding antibiotic resistance genes using the annotation tool of the Antibiotic Resistance Genes Database (ARDB; http://ardb.cbcb.umd.edu/blast/genome.shtml).

Statistical analysis

Each experiment was done in triplicate (biological replicates). Statistical analysis was performed using R software (35). The differences in adhesion and internalization capacities among strains were assessed using one-way analysis of variance. Strains were then grouped using the Tuckey's range test.

Results

Isolation and identification of Lactic Acid Bacteria within bovine mammary ecosystem. Sampling teat canal of 20 cows in two farms led to the isolation and identification of 165 isolates. To avoid redundancy, a selection of these isolates was carried out considering only one clone per species and per cow and ended up with a panel of 76 isolates. These isolates mainly corresponded to LAB, and included enterococci (28.9 %), streptococci (28.9 %), lactobacilli (22.4 %), and lactococci (6.6 %). The remaining isolates corresponded to enterobacteria (9.2%) and staphylococci (3.9%), which are not inhibited on the elective media used (see table 1 in supplemental data). Identification of these isolates based on 16S RNA analysis revealed that streptococcal and enterococcal isolates mainly corresponded to species commonly associated to bovine mastitis (*S. infantarius* and *S. uberis*) (1, 36, 37) and to species associated with fecal flora, respectively. We thus focused on the 22 isolates belonging to *Lactococcus* and *Lactobacillus* genera, which include 1 *L. lactis*, 4 *Lactococcus garvieae*, 4 *Lactobacillus brevis*, 11 *Lactobacillus plantarum* and 2 *L. casei* isolates. At this step, the probiotic strain *L. casei* BL23 was also added to the panel as a reference strain as this strain was previously shown to exhibit probiotic properties (38, 39) and we recently demonstrated that this strain was able to inhibit *S. aureus* internalization into bovine MEC (17).

The 22 LAB isolates and *L. casei* BL23 were then characterized by PFGE (Figure S1). All four *L. garvieae* isolates belonged to the same cluster and similarly, the 2 *L. casei* isolates had the same PFGE profile. The 11 *L. plantarum* isolates fell into 7 groups with unique PFGE profiles and the 4 *L. brevis* isolates corresponded to 3 unique PFGE profiles. Characterization of the beneficial properties was then done on 10 arbitrarily selected isolates corresponding to unique PFGE profiles (i.e. corresponding to unique strains), to avoid any risk of redundancy. The actual panel of strains thus includes 1 *L. lactis*, 1 *L. garvieae*, 3 *L. brevis*, 4 *L. plantarum*, and 1 *L. casei* isolates (in addition to *L. casei* BL23, used as control) (Table 1).

Characterization of inhibitory potential against pathogenic bacteria. None of the tested strains was found to produce hydrogen peroxide, as measured by the colorimetric method on TMB agar plates (Table 1). Production of inhibitory compounds in the supernatant was tested using the agar plate diffusion method (see Materials and methods for details). Untreated supernatants of 7 strains were able to inhibit, at least partially, the indicator strains. All *L. casei* and *L. plantarum* strains inhibited growth of all the indicator strains except that of *S. aureus* N305. On the contrary, no growth inhibition was observed for the *L. garvieae* and *L. brevis* strains tested. An intermediate inhibitory capacity was observed for *L. lactis* 1596. Inhibition was relieved in all cases when supernatants were neutralized with NaOH.

Surface properties of LAB isolated from the bovine mammary ecosystem.

A great majority of strains (8 out of 11) exhibited a low hydrophobicity, while no strain was classified as highly hydrophobic. Three strains had medium hydrophobicity, namely *L. plantarum* 1610 and *L. brevis* 1595 and 1597. Autoaggregation capacities were low for all strains but one, *L. brevis* 1597, which had a medium autoaggregation capacity.

Colonization potential of LAB.

Adhesion capacities of LAB were highly strain-dependent, with differences between strains of up to ~1.6 LOG_{10}, independently of the ROI (Figure 1). Inter- and intra-species variability was observed as illustrated for *L. brevis* and *L. plantarum*. Hence, for a ROI of 400:1, adhesion rate of *L. brevis* 1613 was 1.2×10^4 cfu per well (corresponding to 2.5×10^5 MAC-T cells) whereas it reached 3.1×10^5 and 2.1×10^5 cfu per well for *L. brevis* 1595 and 1597 respectively. Likewise, the adhesion rate of *L. plantarum* 1610 was 5×10^5 cfu per well whereas it was ~2.7×10^4 cfu per well for *L. plantarum* 1601 and 1612. Two strains, *L. brevis* CIRM-BIA 1595 and *L. plantarum* CIRM-BIA 1610, exhibited adhesion capacities

significantly higher than that of the others for a ROI of 400:1. *L. brevis* CIRM-BIA 1597 and *L. plantarum* CIRM-BIA 1602 exhibited intermediate adhesion capacities. On the contrary, *L. lactis* 1596, *L. garvieae* 1605, *L. brevis* CIRM-BIA 1613, the two *L. casei* strains tested (including strain BL23) and *L. plantarum* 1612 had significantly lower adhesion capacities. These trends were confirmed at a ROI of 2000:1 albeit with attenuation.

Internalization capacities of LAB were also highly strain-dependent, with differences of up to 3 LOG_{10}, independently of the ROI (Figure 2). Three strains, *L. brevis* CIRM-BIA 1595 and 1597 and *L. plantarum* CIRM-BIA 1610, exhibited internalization capacities significantly higher than that of the others for a ROI of 400:1. Hence, internalization rates of *L. brevis* 1595, 1597 and *L. plantarum* 1610 at ROI 400:1 were 5.9×10^4 , 4.7×10^4 and 1.0×10^5 cfu per well respectively, whereas it was between 1.5×10^2 and 5.0×10^3 cfu per well for the other strains. Differences were strongly attenuated at a ROI of 2000:1 as only *L. brevis* CIRM-BIA 1595 internalized at a significantly higher rate compared to the others.

Of note, the cellular layer integrity was not affected by incubation with any of the LAB in the conditions used: the MAC-T cells population kept constant at 2.5×10^5 cells per well and the cellular layer did not exhibit any changes in cell morphology during the time of experiment (not shown).

Characterization of the immunomodulatory potential of selected LAB

Screening of LAB isolates on the HT29 and PBMC models (see material and methods for full details) revealed a strain-dependent capacity of these LAB to stimulate the production of a pro-inflammatory interleukin, IL-8, and an anti-inflammatory interleukin, IL10 respectively. *L. garvieae* 1605, *L. casei* 1542, and, to a lesser extent, *L. brevis* 1595, were shown to significantly stimulate the production of IL8 in HT29 cells. Hence, IL8 production was 1.4-fold higher in the presence of *L. garvieae* 1605, *L. casei* 1542, and only 1.1-fold higher in the

presence of *L. brevis* 1595, compared to IL8 production in the reference condition (HT29 cells stimulated with TNF-α alone). Using the PBMC model, production of IL10 was significantly lower in the presence of *L. garvieae* 1605, *L. casei* BL23 and *L. plantarum* 1610 than with *L. rhamnosus* LGG, known to stimulate IL10 production in this model.

Genome sequencing and identification of proteins potentially exposed at the cell surface and of potential antibiotic resistance gene.

Total genomes of 5 out of the 10 LAB that were characterized, namely *L. brevis* 1595, *L. casei* 1542, *L. lactis* 1596, and *L. plantarum* 1610 and 1612, were determined leading to the identification of 2429, 2760, 2339, 3091 and 3038 CDS respectively. These strains were selected to include strains corresponding to all species except *L. garvieae*, exhibiting different colonization and immunomodulation properties. *L. garvieae* was excluded at this step as its safety status may be questioned (40). Combining annotation of proteins with their localization (PSE and secreted proteins) as well as the presence of specific conserved domain allowed us to establish a list of proteins potentially involved in tissue colonization through their binding to cells or extracellular matrix (collagen, mucin, fibronectin) (Table S1). All LAB strains, including BL23, encode three to six proteins containing a collagen-binding domain and one or two proteins containing a fibronectin-binding domain. All strains contain sortases, with up to 4 sortases for *L. casei* strains. On the contrary, strain-to-strain variations occurred in the genomic content for MucBP domains, with three to four proteins with a MucBP domain for *L. brevis* 1595 and *L. plantarum* strains, one for *L. lactis* 1596 and no protein with MucBP domain in *L. casei* strains. Besides, potential S-layer proteins were only found in *L. brevis* 1595 genome, whereas genes potentially involved in capsular polysaccharide biosynthesis were present in *L. casei* 1542, *L. lactis* 1596 and *L. plantarum* strains. All strains were also

found to possess one or more proteins annotated as Internalin J precursor. However, the conserved domain content strongly varies between these proteins (Table S1).

The presence of potential antibiotic resistance genes was checked, revealing only few potential antibiotic resistance genes. Indeed, no gene encoding antibiotic resistance genes was found in *L. brevis* 1595. All strains but one (*L. brevis* 1595) encode a potential bacitracin resistance gene. Finally, *L. lactis* 1596 was found to carry two additional genes potentially coding for fluoroquinolone and tetracycline resistance.

Discussion

In this study, we investigated 10 LAB strains isolated from the bovine mammary microbiota with regard to their capacity to colonize the mammary gland epithelium, their inhibitory potential on the 3 main etiological agents of bovine mastitis, and their capacity to stimulate the innate immune system. In addition, the genomes of 5 out of these 10 strains were fully sequenced, revealing interesting genetic determinants potentially linked to the phenotypes observed.

Diversity of LAB within the bovine mammary microbiota. The bovine mammary microbiota has previously been investigated through phylogenomic approaches and showed that Firmicutes are among the dominant phyla, *Staphylococcus* and *Enterococcus* as the dominant genera (41, 42). Accordingly, in this study, using elective media for LAB, we isolated 165 clones from the bovine mammary microbiota, and identified 76 clones, of which ~29% were enterococci (*Enterococcus faecium, E. hirae*). Another ~29 % belonged to *Streptococcus* genus (*Streptococcus infantarius* and *S. uberis*). These species have previously been isolated from foremilk samples and are reportedly associated to mastitis and or fecal contamination (11, 43). We focused our study on the LAB strains belonging to *Lactococcus* (6.6% of the isolates) and *Lactobacillus* (22.4%) genera because some species in these genera have a GRAS status and some strains are already used as probiotics in other context. The 22 strains isolated grouped into 14 PFGE profiles, with heterogeneity (two or more profiles) in most of the species investigated here, except for *L. garvieae* whose 4 strains were homogenous and grouped in one single PFGE profile. Based on these PFGE results, ten non-redundant LAB strains were selected and characterized in depth, together with the probiotic strain *L. casei* BL23. The genomes of 5 of these strains (*L. lactis* 1596, *L. plantarum* 1610 and 1612, *L. brevis* 1595, and *L. casei* 1542) were fully sequenced, which allowed the identification of genetic determinants potentially involved in the phenotypes observed.

Besides, it showed that the selected strains harbored only few antibiotic resistance genes with resistance to bacitracine common to all five strains. *L. lactis* presented an additional two resistance determinants against tetracycline and fluoroquinolone, antibiotics widely used in mastitis treatment (Table S2).

Surface properties of mammary LAB strains and colonization. One important trait for probiotics is the ability to compete with pathogens for the niche. We thus characterized the LAB strains with regards to their inhibitory potentials in terms of acidification, bacteriocin-like compounds, and hydrogen peroxide and with regards to their colonization capacity.

The ability of the 10 LAB isolates to inhibit growth of representative strains of *E. coli*, *S. uberis* and *S. aureus*, the three major pathogens involved in bovine mastitis, was highly species-specific. Supernatants of *L. plantarum* and *L. casei* strains showed the highest inhibitory potential (inhibition of the 3 pathogens), as well as the lowest pH values. On the other hand, supernatants of the *L. brevis* strains and *L. garvieae* 1605 had a moderate pH (final pH >5) and showed a poor inhibition. The inhibition observed exclusively relied on acidification since neutralization of the supernatants totally relieved their inhibitory activity. None of our 10 LAB strains were found to produce hydrogen peroxide. Hydrogen peroxide production is considered an interesting traits of vaginal probiotic LAB (44, 45). It was also previously reported for some (but not all) LAB strains isolated from mammary microbiota although with a lower frequency than in vaginal LAB (11, 43, 46). This might reflect a different adaptation of LAB strains within mammary and vaginal ecosystems.

The ability to colonize tissues and, as a result, exert a prolonged beneficial effect and or compete with pathogens for the niche is one of the criteria used to select a candidate probiotic strain. Capacities of the 10 selected LAB to colonize of the mammary gland was investigated in vitro in adhesion and internalization assays using bMEC.

Our results clearly showed strain-to-strain variations of adhesion capacities of LAB isolates, and *L. brevis* 1595 and 1597, and *L. plantarum* 1610 harbored the strongest adhesion to bMEC. These strains were also those which had the highest hydrophobicity. Such correlation between adhesion and hydrophobicity had previously been reported (43, 47). Interestingly, one strain with high adhesion capacity, namely *L. brevis* 1597, exhibited auto-aggregation property. Autoaggregation is thought to favor formation of protective biofilm (46). Auto-aggregative strains also present another interest as they may titer pathogenic microorganisms by co-aggregation and facilitate its clearance (48) .

Genomic comparison of the two strains exhibiting the highest adhesion rates (*L. brevis* 1595 and *L. plantarum* 1610), and those exhibiting intermediate (*L. plantarum* 1612) or low adhesion rates (*L. lactis* 1596, *L. casei* 1542 and *L. casei* BL23) (Table S1) revealed strong differences in terms of genes potentially involved in tissue colonization. Such variations in adhesion determinants were already reported in LAB and include several proteins directly involved in adhesion to mucus, fibronectin or collagen, S-layer proteins or proteins involved in capsular polysaccharide biosynthesis, as well as some house-keeping gene products (8, 9, 49–51). Here, three to four MucBP domain proteins were found in strains exhibiting high or intermediate adhesion rates, whereas only one MucBP domain protein was found in *L. lactis* 1596 and none were present in *L. casei* genomes. Moreover, only *L. brevis* 1595 encodes S-layer proteins. Besides, it is noticeable that the three highly adhesive strains, i.e. *L. brevis* 1595 and the two *L. plantarum* strains, possess two potential fibronectin-binding proteins, carrying the conserved domains FNB and FnbA respectively, while the low adhesive strains only possess one copy of FbpA-domain protein. Whether the variations in adhesion capacities we observed in this study are linked to the presence or absence of one or several of the above mentioned adhesion determinants remains to be explored.

Studies investigating the capacity of LAB to colonize host tissue are generally restricted to the evaluation of their adhesion properties, with the aim to prevent the first step of tissue colonization by pathogens. The microbial capacity to internalize and, possible, survive and proliferate within cells is still associated to pathogenic bacteria and, as a result, not explored for the so-called "positive bacteria". Hence, only a few studies report the internalization of LAB and with the aim to use LAB as vehicles for intracellular delivery of molecules (52, 53). In this study, the capacity of LAB to internalize was investigated. As observed with adhesion, the ability of LAB strains to internalize was strongly strain-dependent. This ability to internalize was related to the ability to adhere, as illustrated by a pearson correlation coefficient of 0.89 between both capacities at a ROI of 400:1. Interestingly, full genome sequencing of the five strains revealed the presence of Internalin-J like proteins, which share conserved domains with *Listeria monocytogenes* Internalin J (54). Of note, despite similarities with Internalin A and B, the exact function of Internalin-J in *L. monocytogenes* virulence is not fully understood yet (54).

Of note, none of the internalized LAB strains induced any alteration of the host cell morphology. Although the capacity of the tested LAB to internalize was limited compared to one of the major etiologic agent of mastitis (*S. aureus*) (17), it raises questions about the possible persistence of LAB in the tissue and their effect on cellular physiology, cell cycle or epigenetic modifications, as observed with pathogenic bacteria, such as *S. aureus* (55, 56). This question needs further investigations.

Immunomodulation by bovine mammary LAB strains. Bovine mastitis is basically an inflammation of the mammary gland. On one hand, a probiotic LAB candidate with a slight pro-inflammatory capacity can be of interest to stimulate the innate immunity and thus to prevent mastitis. On the other hand, a candidate strain with an anti-inflammatory capacity would help resolving the inflammation in infectious mastitis and help the return to lactation.

Characterization of the immunomodulatory properties of the LAB isolates revealed that *L. casei* 1542 and *L. garvieae* 1605 exhibited pro-inflammatory properties, as illustrated by the stimulation of pro-inflammatory cytokine IL8 production in HT29. *L. garvieae* 1605 also showed a low capacity to stimulate the anti-inflammatory cytokine IL10 production by PBMC. Likewise, *L. plantarum* 1610 poorly stimulated the production of IL10 when compared to the well-known stimulator of IL10 production strain, *L. rhamnosus* LGG.

Some of the above-mentioned determinants that are putatively involved in adhesion and internalization have also been correlated with the immunomodulation properties of LAB (9, 51). The involvement of these determinants in the observed immunomodulation properties will deserve further experiments.

In conclusion, we selected three LAB strains based on their potential to colonize mammary gland tissue and their pro -inflammatory properties. Some strains present high colonization capacity, and are good candidate to compete with pathogens for the mammary gland tissue and to exert a prolonged beneficial effect. Pro-inflammatory properties could help stimulating the innate immune system and promote clearance of pathogens. Anti-inflammatory properties could contribute to the decrease of inflammation in association to, or following an antibiotic treatment. Presence of risk factors such as antibiotic resistance genes was also checked in our screening in order to prevent the risk of antibiotic resistance dissemination. These candidate strains require further investigation to evaluate their barrier effect with regard to major mastitis pathogens and their immune-modulatory potential on bovine mammary epithelial cells. The ultimate tests will of course have to be carried out in vivo, first to test their safety, and second to challenge their efficacy in field conditions.

Figure legends

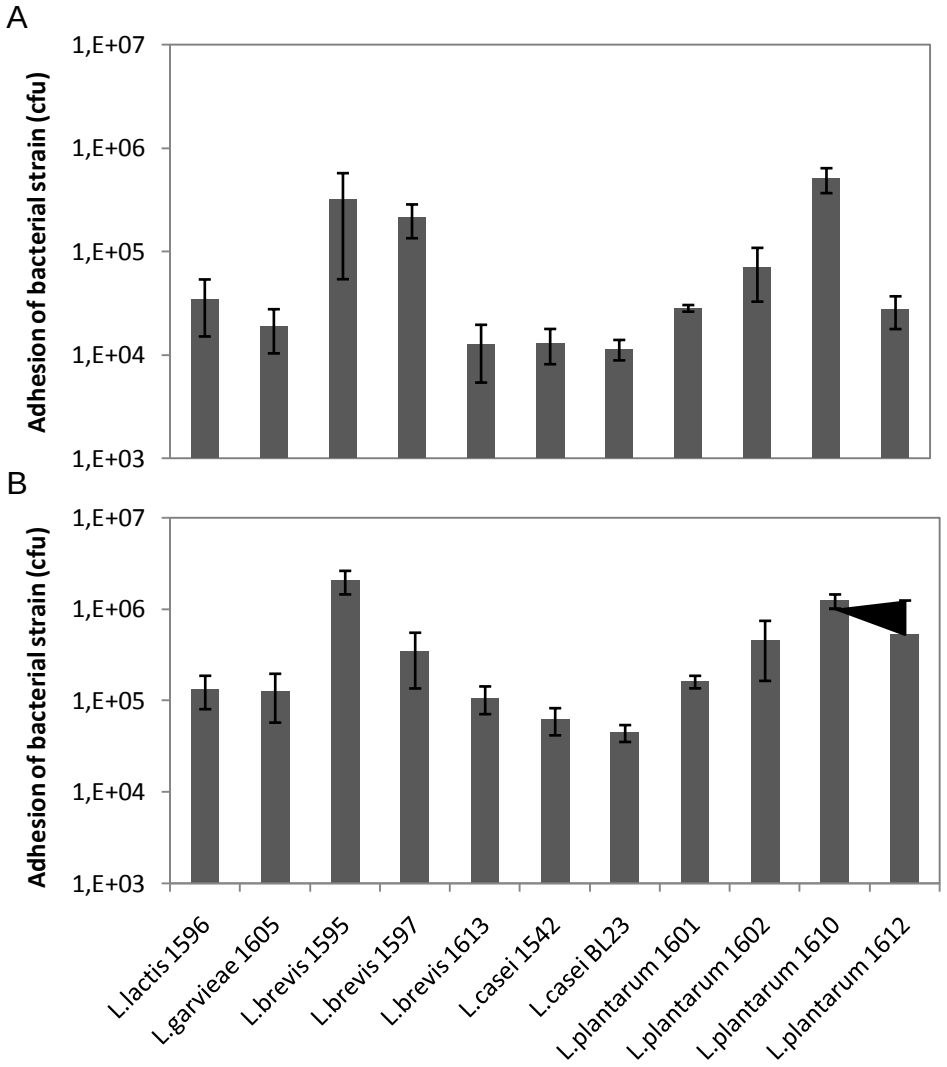

Figure 1. Adhesion of lactic acid bacteria to bovine mammary epithelial cells.

Lactic acid bacteria populations adhered to bMEC were determined after 1 h of interaction at a ROI of 400:1 (A) and 2000:1 (B) respectively. Data are presented as mean population per well (i.e., corresponding to 2.5. 10^5 bMEC) +/- Standard Deviation. Each experiment was done in triplicate and differences between strains were assessed using a one-way analysis of variance, followed by a Tukey's range test. Letters a, b, c and d indicate homogeneous statistical processing groups that were significantly different according to the Tukey's range test.

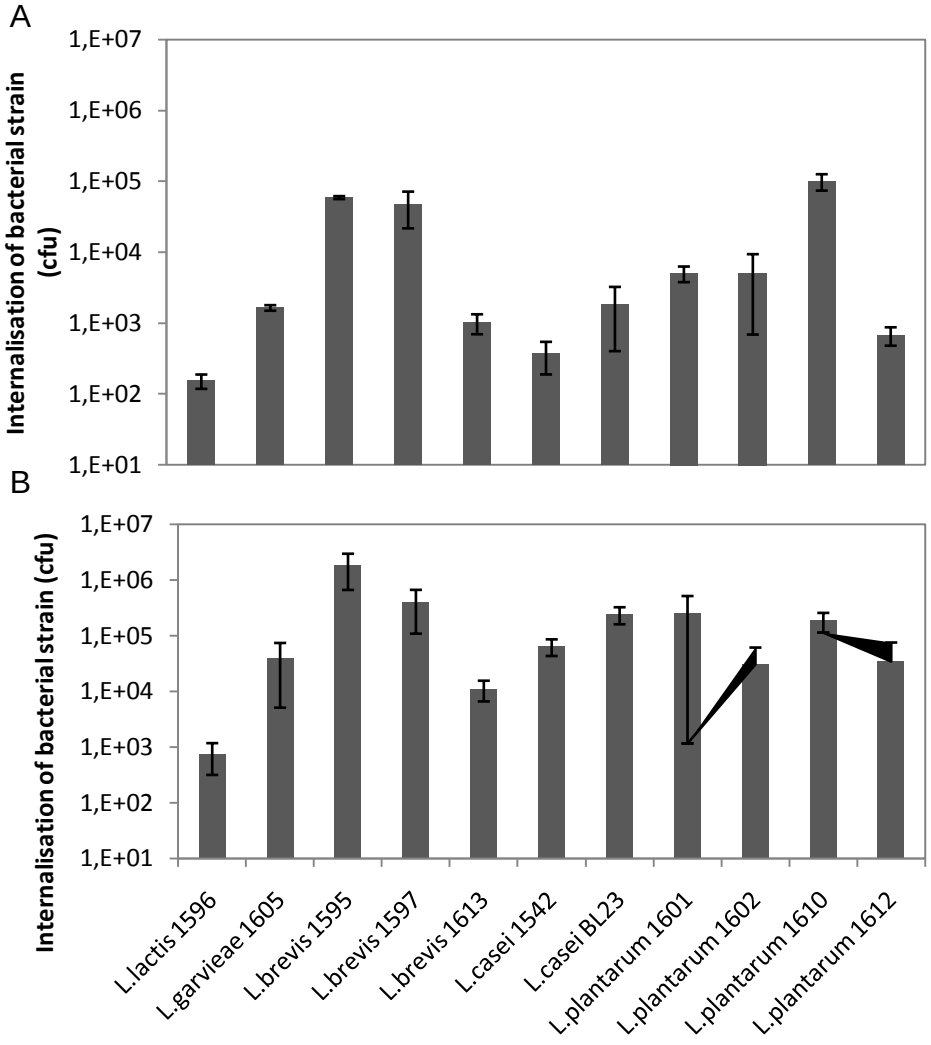

Figure 2. Internalization of lactic acid bacteria into bovine mammary epithelial cells.

Lactic acid bacteria populations internalized into bMEC were determined after 2 h of interaction at a ROI of 400:1 (A) and 2000:1 (B) respectively. Data are presented as mean population per well (i.e., corresponding to 2.5. 10^5 bMEC) +/- Standard Deviation. Each experiment was done in triplicate and differences between strains were assessed using a one-way analysis of variance, followed by a Tukey's range test. Letters a, b, c and d indicate homogeneous statistical processing groups that were significantly different according to the Tukey's range test.

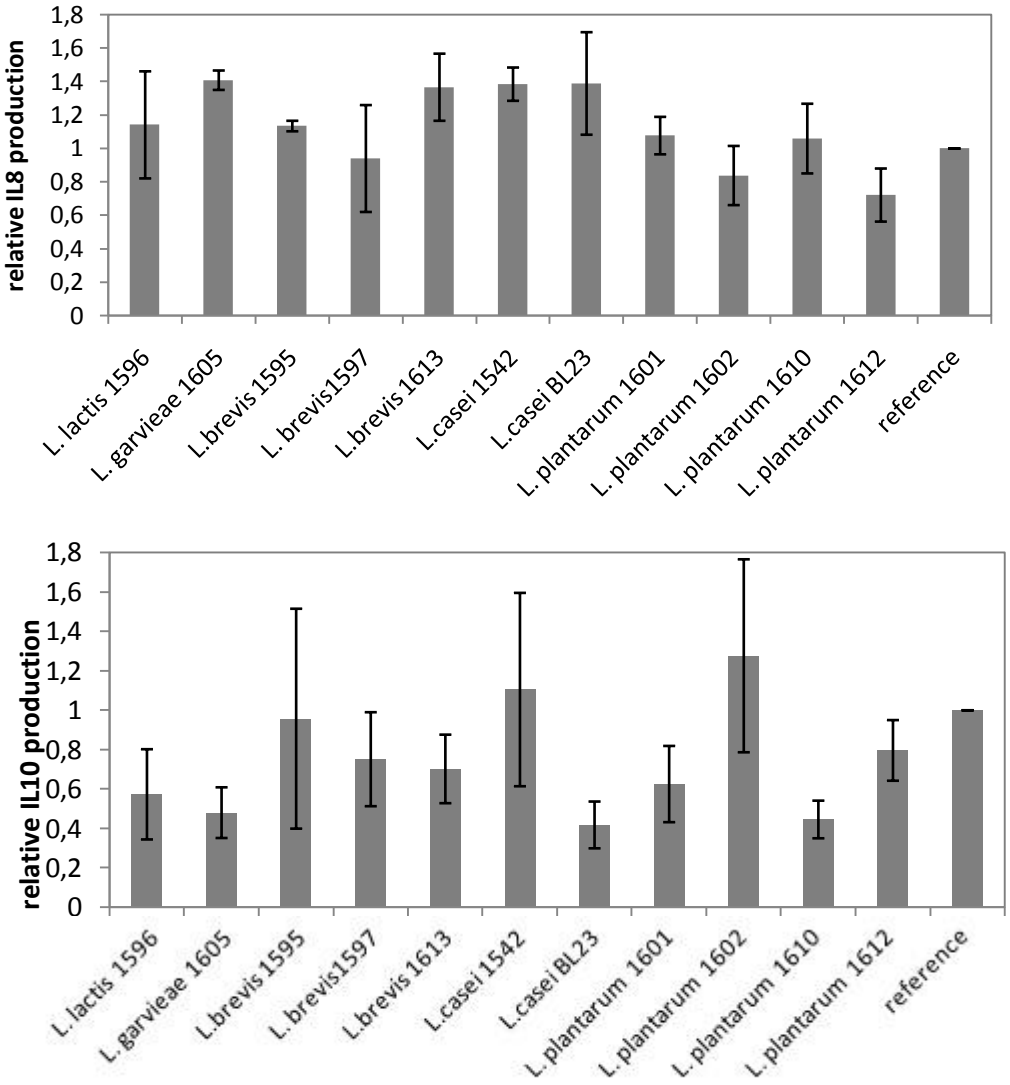

Figure 3. Modulation of cytokine production by LAB isolates. A: modulation of IL8 production by HT29 cell line in the presence of LAB isolates. Bars represent the mean IL8 production ± standard deviation for 3 independent assays, normalized with regard to IL8 production when stimulation of HT29 cell line was done with TNF-α alone (reference condition). B: modulation of IL10 production by human PBMC in the presence of LAB isolates. Bars represent the mean IL10 production ± standard deviation for 3 independent healthy donors, normalized with regard to IL10 production in the presence of *L. rhamnosus* LGG (known to induce IL10 production, reference condition).

Differences in IL8 and IL10 production with regard to the reference condition was assessed using a one-way analysis of variance (* pval < 0.05).

Table 1. Characterization of surface and antagonistic properties of LAB strains isolated from bovine teat canal

Name		sample type	Surface properties				Antimicrobial properties			
			Hydrophobicity		Autoaggregation		H2O2 production	acidification	diffusion test	
Species	CIRM-BIA		% [a]	Gr [b]	% [c]	Gr [d]	TMB test [e]	pH SN [f]	native SN [g]	neutralized SN [g]
Lactococcus lactis	1596	Foremilk	21	L	10	L	NP	4.28	*S. aureus* RF122 / NB305 *E. coli* LMA1678 /LMA1674	-
Lactococcus garvieae	1605	Swab	7	L	7	L	NP	4.45	-	-
Lactobacillus brevis	1613	Foremilk	25	L	8	L	NP	5.25	-	-
Lactobacillus brevis	1595	Foremilk	46	M	15	L	NP	5.23	-	-
Lactobacillus brevis	1597	Swab	35	M	66	M	NP	5.09	-	-
Lactobacillus plantarum	1610	Foremilk	60	M	8	L	NP	3.89	*S. aureus* RF122 *E. coli* LMA1678/LMA1674 *S. uberis* LMA1675/LMA1672	-
Lactobacillus plantarum	1612	Foremilk	0	L	13	L	NP	3.84	*S. aureus* RF122 *E. coli* LMA1678/LMA1674 *S. uberis* LMA1675/LMA1672	-
Lactobacillus plantarum	1602	Foremilk	7	L	11	L	NP	3.93	*S. aureus* RF122 *E. coli* LMA1678/LMA1674 *S. uberis* LMA1675/LMA1672	-
Lactobacillus plantarum	1601	Swab	5	L	14	L	NP	3.92	*S. aureus* RF122 *E. coli* LMA1678/LMA1674 *S. uberis* LMA1675/LMA1672	-
Lactobacillus casei [n]	1542	Swab	6	L	14	L	NP	4.10	*S. aureus* RF122 *E. coli* LMA1678/LMA1674 *S. uberis* LMA1675/LMA1672	-
Lactobacillus casei BL23			10	L	13	L	NP	4.17	*S. aureus* RF122 *E. coli* LMA1678/LMA1674 *S. uberis* LMA1675/LMA1672	-

Supplementary material

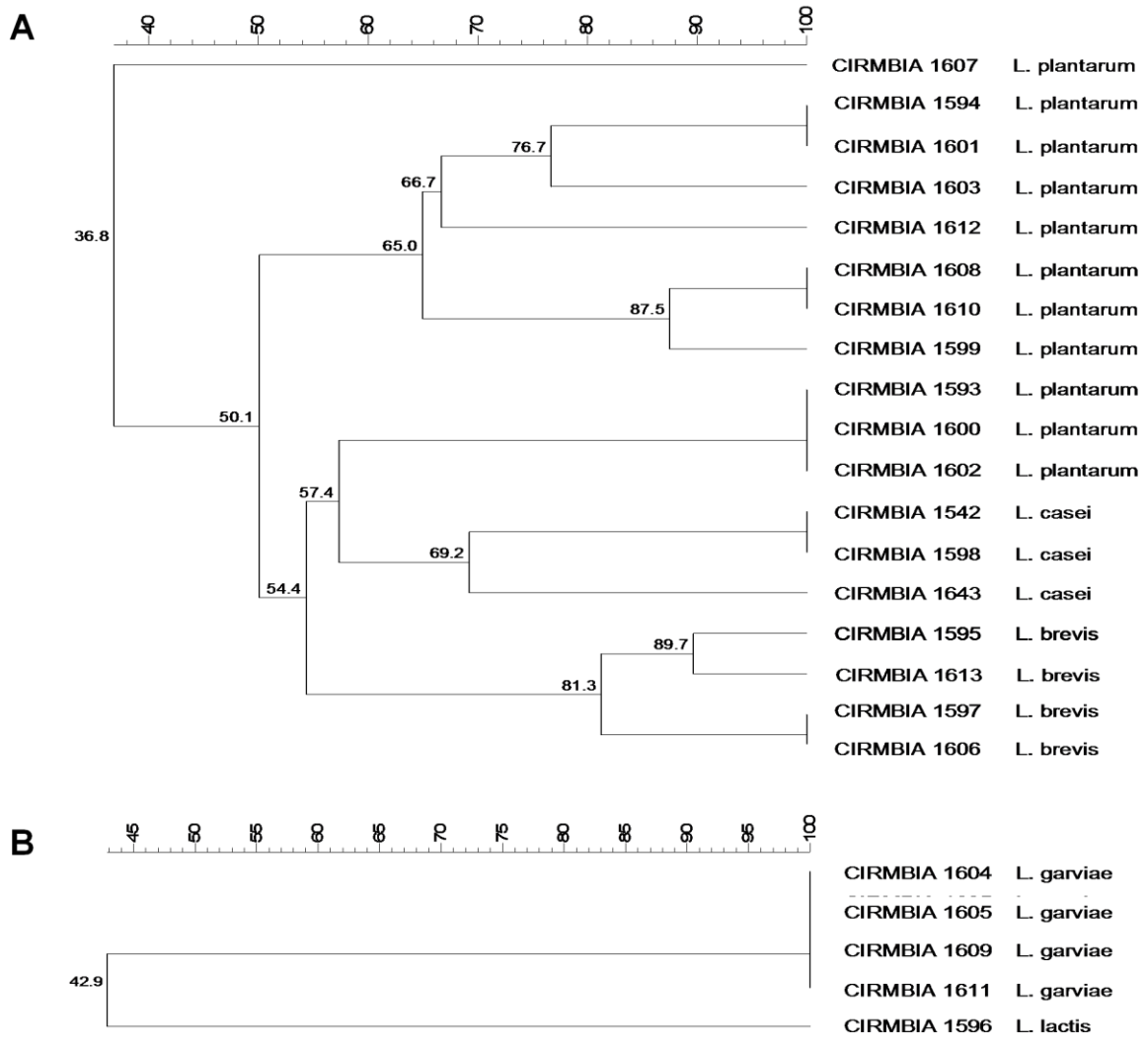

Figure S1. Dendograms of PFGE patterns of *Lactobacillus* sp. (A) obtained with endonuclease AscI and *Lactococcus* sp. (B) with endonuclease SmaI. The similarities of the profiles were calculated using Dice coefficient and dendograms were obtained by UPGMA clustering algorithm.

Table S1. Potential bacterial determinants of LAB colonization capacities and immunomodulation properties in *L. brevis* 1595, *L. casei* 1542, *L. lactis* 1696, *L. plantarum* 1610 and 1612 and *L. casei* BL23

ID	Description	Conserved Domains [a]	Prediction [b]	Length	signal peptide [b]	Comment [b]
***L. brevis* 1595**						
lactobrevis_1595_01027	Cna protein B-type domain protein	Cna-B, collagen_bind superfamily	PSE	619	Y	LPxTG
lactobrevis_1595_01481	Cna protein B-type domain protein	Cna-B, collagen_bind superfamily	PSE	663	Y	LPxTG
lactobrevis_1595_00487	Collagen binding domain protein	collagen_bind superfamily	PSE	443	Y	
lactobrevis_1595_01650	Internalin-J precursor	DUF285, Big_3 superfamily (Ig-like domain)	SECRETED	648	Y	
lactobrevis_1595_01722	Internalin-J precursor	MucBP, LRR_4, LRR_8	PSE	912	Y	LPxTG
lactobrevis_1595_01290	MucBP domain protein		PSE	422	N	
lactobrevis_1595_01879	MucBP domain protein	MucBP	PSE	1519	Y	LPxTG
lactobrevis_1595_02463	MucBP domain protein	MucBP	PSE	1111	Y	LPxTG
lactobrevis_1595_02534	MucBP domain protein		PSE	454	N	LPxTG
lactobrevis_1595_01519	S-layer protein precursor		SECRETED	470	Y	
lactobrevis_1595_02073	S-layer protein precursor		SECRETED	413	Y	
lactobrevis_1595_01520	S-layer protein precursor		PSE	457	Y	
lactobrevis_1595_02440	S-layer protein precursor		PSE	427	Y	
lactobrevis_1595_01876	Sortase family protein		PSE	237	Y	
lactobrevis_1595_00365	Fibronectin-binding protein	FBP	CYTOPLASMIC	215	N	
lactobrevis_1595_00598	Fibronectin-binding protein	FbpA	CYTOPLASMIC	568	N	
***L. casei* 1542**						
lactocasei_1542_01840	Capsular polysaccharide type 8 biosynthesis protein cap8A		PSE	312	N	
lactocasei_1542_02809	Capsular polysaccharide type 8 biosynthesis protein cap8A		PSE	309	Y	

ID	Description	Conserved Domains [a]	Prediction [b]	Length	signal peptide [b]	Comment [b]
***L. casei* 1542 (suite)**						
lactocasei_1542_00868	Cna protein B-type domain protein		PSE	334	Y	LPxTG
lactocasei_1542_00986	Cna protein B-type domain protein	Cna-B	PSE	1269	Y	LPxTG

lactocasei_1542_01071	Cna protein B-type domain protein	Cna-B	PSE	1001	Y	LPxTG
lactocasei_1542_01015	Cna protein B-type domain protein	collagen_bind superfamily, Cna-B	PSE	611	Y	
lactocasei_1542_02086	Cna protein B-type domain protein	Cna-B	PSE	2724	Y	
lactocasei_1542_01073	Fimbrial subunit type 1 precursor	Cna-B	PSE	519	Y	LPxTG
lactocasei_1542_01105	Internalin-J precursor		SECRETED	192	Y	
lactocasei_1542_00689	Internalin-J precursor		PSE	230		LPxTG
lactocasei_1542_02428	Sortase family protein		SECRETED	296	Y	
lactocasei_1542_00867	Sortase family protein		PSE	358	Y	
lactocasei_1542_01074	Sortase family protein		PSE	275	Y	
lactocasei_1542_02561	Sortase family protein		PSE	233		
lactocasei_1542_00870	von Willebrand factor type A domain protein	Cna-B, collagenBindB superfamily	PSE	909	Y	LPxTG
lactocasei_1542_00633	Hypothetical protein (FbpA domain)	FbpA	CYTOPLASMIC	567	N	
***L. lactis* 1596**						
lactolactis_1596_00950	Capsular polysaccharide type 8 biosynthesis protein cap8A		PSE	259	Y	
lactolactis_1596_00957	Capsular polysaccharide type 8 biosynthesis protein cap8A		PSE	191	N	
lactolactis_1596_01025	Cna protein B-type domain protein	Collagen_bind, Cna-B	PSE	1983	Y	LPxTG
lactolactis_1596_01964	Cna protein B-type domain protein		PSE	614	Y	LPxTG
lactolactis_1596_00248	Collagen adhesin precursor	collagenBindB superfamily	PSE	822	Y	
lactolactis_1596_01240	Collagen adhesin precursor	Collagen_bind, collagenBindB superfamily	PSE	366		LPxTG
lactolactis_1596_2132	Serine-rich adhesin for platelets precursor	MucBP	PSE		Y	LPxTG

ID	Description	Conserved Domains [a]	Prediction [b]	Length	signal peptide [b]	Commen [b]
***L. lactis* 1596 (suite)**						
lactolactis_1596_02063	Internalin-J precursor		SECRETED	338	Y	
lactolactis_1596_00327	Sortase family protein		PSE	248	Y	
lactolactis_1596_01963	Sortase family protein		PSE	431	Y	
lactolactis_1596_02139	Fibronectin-binding protein	FBP superfamily	CYTOPLASMIC	218	N	
***L. plantarum* 1610**						
lactoplantarum_1610_02069	Capsular polysaccharide phosphotransferase cps12A		PSE	321	N	
lactoplantarum_1610_00571	Capsular polysaccharide type 8 biosynthesis protein		PSE	252	N	

	cap8A					
lactoplantarum_1610_03115	Capsular polysaccharide type 8 biosynthesis protein cap8A		PSE	256	Y	
lactoplantarum_1610_02763	Cna protein B-type domain protein	collagen_bind superfamily, Cna-B	PSE	647	Y	
lactoplantarum_1610_01581	Collagen binding domain protein	collagen_bind superfamily	PSE	617	Y	
lactoplantarum_1610_01448	Internalin-J precursor	Liste_lipo_26, DUF285	SECRETED	750	Y	
lactoplantarum_1610_01441	Internalin-J precursor	LRR_4, LRR_8	PSE	1189	Y	LPxTG
lactoplantarum_1610_01827	Internalin-J precursor		PSE	1260	Y	LPxTG
lactoplantarum_1610_02840	Internalin-J precursor		PSE	906	Y	LPxTG
lactoplantarum_1610_00178	MucBP domain protein	MucBP, MucBP superfamily	PSE	2217	Y	LPxTG
lactoplantarum_1610_01451	MucBP domain protein	MucBP, MucBP superfamily	PSE	2023	Y	LPxTG
lactoplantarum_1610_03092	MucBP domain protein	MucBP, MucBP superfamily	PSE	252		LPxTG
lactoplantarum_1610_01497	IgA FC receptor precursor	MucBP superfamily	PSE		Y	LPxTG
lactoplantarum_1610_00912	Agglutinin receptor precursor	collagenBindB superfamily	PSE	994	Y	
lactoplantarum_1610_00657	Sortase family protein		SECRETED	234	Y	

ID	Description	Conserved Domains [a]	Prediction [b]	Length	signal peptide [b]	Commen [b]
***L. plantarum* 1610 (suite)**						
lactoplantarum_1610_01031	Fibronectin-binding protein	FBP, FBP superfamily	CYTOPLASMIC	215	N	
lactoplantarum_1610_00314	Hypothetical protein (FbpA domain)	FbpA	CYTOPLASMIC	568	N	
L. plantarum 1612						
lactoplantarum_1612_02954	Capsular polysaccharide type 8 biosynthesis protein cap8A		PSE	255	N	
lactoplantarum_1612_03033	Capsular polysaccharide type 8 biosynthesis protein cap8A		PSE	256	N	
lactoplantarum_1612_00297	Agglutinin receptor precursor	collagenBindB superfamily, Cna-B	PSE	1038	Y	
lactoplantarum_1612_1695	Agglutinin receptor precursor	Cna-B, collagen_bind superfamily	PSE	724	Y	LPxTG
lactoplantarum_1612_02566	Collagen binding domain protein	collagen_bind superfamily	PSE	617	Y	
lactoplantarum_1612_01701	Immunoglobulin G-binding protein A precursor	collagen_bind superfamily	PSE	554	Y	LPxTG
lactoplantarum_1612_02471	Internalin-J precursor	LRR_4,LRR_8	PSE	1194	Y	LPxTG

lactoplantarum_1612_02415	IgA FC receptor precursor (MucBP domain)	MucBP, MucBP superfamily	PSE	1369	Y	LPxTG
lactoplantarum_1612_01057	MucBP domain protein	MucBP, MucBP superfamily	PSE	2217	Y	LPxTG
lactoplantarum_1612_02461	MucBP domain protein	MucBP, MucBP superfamily	PSE	2032	Y	LPxTG
lactoplantarum_1612_00039	Sortase family protein		SECRETED	234	Y	
lactoplantarum_1612_00426	Fibronectin-binding protein	FBP, FBP superfamily	CYTOPLASMIC	215	N	
lactoplantarum_1612_00918	Hypothetical protein (FbpA domain)	FbpA	CYTOPLASMIC	568	N	
L. casei BL23						
gi|191637338|ref|YP_001986504.1|	hypothetical protein LCABL_05200	Cna-B,collagenBindB superfamily	PSE	909	Y	LPxTG
gi|191639298|ref|YP_001988464.1|	hypothetical protein LCABL_25400	Cna-B	PSE	519	Y	LPxTG
gi|191639300|ref|YP_001988466.1|	outer membrane protein	Cna-B	PSE	1001	Y	LPxTG

ID	Description	Conserved Domains [a]	Prediction [b]	Length	signal peptide [b]	Commen [b]
L. casei BL23 (suite)						
gi|191639361|ref|YP_001988527.1|	outer membrane protein	Cna-B,collagen_bind superfamily	PSE	611	Y	
gi|191639436|ref|YP_001988602.1|	outer membrane protein	Cna-B	PSE	2726	Y	
gi|191639856|ref|YP_001989022.1|	Adhesion exoprotein		PSE	672	N	LPxTG
gi|191637480|ref|YP_001986646.1|	Collagen binding protein	SBP-bac-3	PSE	270	Y	
gi|191637340|ref|YP_001986506.1|	Fimbriae subunit		PSE	334	Y	LPxTG
gi|191639262|ref|YP_001988428.1|	Internalin-J		PSE	423	Y	LPxTG
gi|191637433|ref|YP_001986599.1|	sortase srtA2 (LCABL_06160)		SECRETED	223	Y	
gi|191639079|ref|YP_001988245.1|	sortase srtA1 (LCABL_23200)		PSE	233	N	
gi|191639297|ref|YP_001988463.1|	sortase srtC1 (LCABL_25390)		PSE	275	Y	
gi|191637341|ref|YP_001986507.1|	sortase srtC2 (LCABL_05230)		PSE	358	Y	
gi|191638435|ref|YP_001987601.1|	Fibronectin-binding protein A	FbpA	CYTOPLASMIC	567	N	

Table S2. Potential antibiotic resistance genes encoded in *L. brevis* 1595, *L. casei* 1542, *L. lactis* 1696, *L. plantarum* 1610 and 1612 and *L. casei* BL23

souche	locus	Type	Definition	Resistance
L. casei 1542	lactocasei_1542_00040	baca	Undecaprenyl pyrophosphate phosphatase, which consists in the sequestration of Undecaprenyl pyrophosphate.	bacitracin
L. lactis 1596	lactolactis_1596_02272	baca	Undecaprenyl pyrophosphate phosphatase, which consists in the sequestration of Undecaprenyl pyrophosphate.	bacitracin
	lactolactis_1596_01002	emea	Major facilitator superfamily transporter. Multidrug resistance efflux pump.	fluoroquinolone
	lactolactis_1596_02421	tets	Ribosomal protection protein, which protects ribosome from the translation inhibition of tetracycline.	tetracycline
L. plantarum 1610	lactoplantarum_1610_01248	baca	Undecaprenyl pyrophosphate phosphatase, which consists in the sequestration of Undecaprenyl pyrophosphate.	bacitracin
L. plantarum 1612	lactoplantarum_1612_02766	baca	Undecaprenyl pyrophosphate phosphatase, which consists in the sequestration of Undecaprenyl pyrophosphate.	bacitracin
L. casei BL23	YP 001986964.1	baca	Undecaprenyl pyrophosphate phosphatase, which consists in the sequestration of Undecaprenyl pyrophosphate.	bacitracin

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Capítulo II

Resumo

A mastite é uma doença inflamatória multifatorial frequentemente associada a uma infecção bacteriana em bovinos de leite que impacta a produção e o beneficiamento do leite, bem como a saúde animal. Por provocar grandes perdas financeiras, a mastite bovina é um importante problema econômico, causando grande impacto para os produtores rurais, a economia industrial beneficiadora do leite e as economias de base agrária como no Brasil e, ainda, regiões de países de economia industrial como a França.

Uma prática largamente difundida nas Américas e Europa para o controle da mastite é o uso da antibioterapia para o tratamento de condições clínicas, bem como, ao final de período de lactação, para a prevenção da condição no rebanho. O uso de antibióticos para o tratamento e para a prevenção da mastite é frequentemente questionado por possibilitar o favorecimento ao surgimento de cepas resistentes às drogas disponíveis no mercado, podendo a mastite tornar se, então, um problema de saúde pública. Por essas razões, a procura por estratégias alternativas são cada vez mais necessárias para prevenir ou curar a mastite em rebanhos acometidos e reduzir o uso de antibióticos de forma maciça em medicina veterinária.

A utilização de probióticos vem aumentando à medida que mais conhecimento vem sendo gerado nessa área e, além de leveduras, as bactérias do ácido lático são frequentemente utilizadas no ambiente intestinal e vaginal. Esse trabalho estuda esse grupo de micro-organismos, reconhecidos como seguros, para uso em seus hospedeiros e que se encontram presentes na microbiota do úbere bovino para sua aplicação no ambiente mamário bovino.

Durante o trabalho de doutorado, foram feitas coletas em duas fazendas leiteiras bovinas do estado de Minas Gerais, Brasil e em 68 bovinos em lactação, durante um ano. Dessas coletas foram isoladas 113 linhagens de bactérias láticas que foram posteriormente identificadas por sequenciamento de DNA 16S. Foram primeiramente selecionadas quatro linhagens do gênero *Lactococcus*, sendo duas *L. lactis* e duas *L. garvieae* em função de

suas propriedades de inibição *in vitro* de *Staphylococcus aureus*, bem como por pertencerem a um gênero reconhecidamente seguro para uso como probiótico. Dessas quatro linhagens foi selecionada a linhagem de *Lactococcus lactis*, denominada V7, por ser capaz de produzir antagonismo contra patógenos e de aderir satisfatoriamente à superfície de células de glândula mamária bovina MAC-T. (dados não publicados).

Lactococcus lactis V7, uma cepa isolada a partir da pele de glândula mamária bovina, foi avaliada como uma opção probiótica para prevenir a mastite bovina. Utilizando-se cultivo de células epiteliais mamárias bovinas (MAC-T), foi demonstrado que *Lactococcus lactis* V7 inibiu a invasão das células por *Escherichia coli* K08 e P4, assim como *S.s aureus* Newbould 305 e RF122. Foi ainda demonstrado que *L. lactis* V7 foi capaz de co-agregar com uma cepa de *E. coli* em suspensão. Essa pode ser uma possível explicação para a inibição da invasão de células mamárias por essa espécie.

Utilizando-se outras células epiteliais mamárias bovinas (PS), a presença de *L. lactis* V7 estimulou a produção de IL-6 e IL-8, tanto em cultivos celulares não infectadas como em cultivos infectados por *E. coli*. *Lactococcus lactis* V7 alterou também a expressão dos genes das interleucinas IL-6 e IL-8, dos receptores *Toll-like* TLR-2 e TLR-4, assim como a expressão do gene de TNF-α nessas mesmas culturas celulares (PS) desafiadas com cepas de *E. coli*. No caso dos genes das interleucinas e de TNF-α houve estimulação pela presença de *L. lactis* V7, enquanto o efeito variou com a expressão dos genes de TLR2 e TLR4 de acordo com as espécies de *E. coli* envolvidas no estímulo. Portanto, *L. lactis* V7 tem a capacidade de alterar a resposta imune de culturas celulares, estimulando a expressão de citocinas que causam a inflamação. Note-se que estas medidas de expressão foram realizadas 24 horas após a estimulação, um tempo relativamente longo em relação a essa estimulação. Experimentos adicionais de cinética em tempos diferentes após a estimulação são, portanto, necessários para esclarecer melhor o efeito de L. lactis V7.

Em conclusão, a cepa *L. lactis* V7, uma bactéria do ácido láctico presente naturalmente na microbiota do úbere bovino, foi capaz de inibir a invasão do tecido mamário em cultivo celular por patógenos de grande importância nas mastites bovinas, além de estimular a

resposta imune das células hospedeiras. Essa cepa apresenta, então, propriedades promissoras para o desenvolvimento de uma estratégia para seu uso como probiótico contra a mastite bovina.

Resumé

La mammite est une maladie inflammatoire multifactorielle souvent associée à une infection bactérienne chez les bovins laitiers. Son impact constitue un problème majeur de santé animale et touche aussi bien la production que la transformation du lait. Les mammites provoquent d'importantes pertes financières et constituent de ce fait un important problème économique, pour les agriculteurs, pour l'économie industrielle du lait et pour les régions productrices comme le Minas Gerais au Brésil et la région Grand Ouest de la France.

Une pratique répandue dans les Amériques et en Europe pour le contrôle de la mammite est l'utilisation d'antibiotiques pour le traitement de manifestations cliniques et à la fin de la période de lactation pour prévenir la maladie dans le troupeau. L'utilisation d'antibiotiques pour le traitement et la prévention de la mammite est souvent questionée en regard des risques d'émergence de souches résistantes aux antibiotiques disponibles sur le marché. La mammite devient alors source de problèmes potentiels en santé publique. Pour ces raisons, la recherche de stratégies alternatives est de plus en plus nécessaire pour prévenir ou guérir la mammite et pour réduire l'utilisation des antibiotiques en médecine vétérinaire.

L'utilisation de probiotiques augmente et de plus en plus de connaissances viennent étayer ce domaine où les bactéries lactiques (BL) sont souvent utilisées dans les sphères digestives et uro-génitales. Les BL sont généralement reconnus comme sûrs (statut GRAS de la FDA) pour l'utilisation dans leurs hôtes et, par ailleurs, elles sont aussi présentes dans le microbiote de la glande mammaire bovine. Cette étude s’intéresse au potentiel probiotique d’une souches de BL dans le cadre de la prévention des mammites bovines.

Pendant les travaux de thèse, des prélèvements ont été faites dans deux élevages bovins laitiers dans l'état de Minas Gerais, au Brésil, soit 68 bovins en lactation, pour un an. De ces échantillons ont été isolés 113 souches de bactéries lactiques qui ont ensuite été identifiés par séquençage de l'ADN 16S. Quatre souches du genre *Lactococcus* ont d'abord été

sélectionnés, deux *L. lactis* et deux *L. garvieae* en fonction de leurs propriétés inhibitrices *in vitro* de *Staphylococcus aureus*, ainsi que l'appartenance à un genre connu pour être sans danger pour une utilisation en tant que probiotique. De ces quatre souches, le *Lactococcus lactis* appelé V7 a été sélectionné, pour être capable d'adhérer de manière satisfaisante à la surface des cellules de la glande mammaire bovine (CEMb) MAC-T. Ces résultats ont fait partie du travail décrit ici, mais ne sont pas présentés dans l'article qui constitue le chapitre deux de cette thèse..

Lactococcus lactis V7, une souche isolée de la peau du trayon, a été évaluée comme une option probiotique pour prévenir la mammite bovine. L'utilisation d'une culture de cellules epitheliales mammaires bovines (MAC-T) a ainsi permis de montrer que *L. lactis* V7 inhibe l'internalisation de *Escherichia coli* K08 et P4 et de *S. aureus* Newbould 305 et RF122 dans les cellules, *in vitro*.

Avec l'aide d'autres CEMb (PS), nous avons montré que la présence de *L. lactis* V7 stimule la production d'IL-8 dans les cultures de cellules non infectées et dans les cultures infectées par *E. coli*. D'autres expérimentations complémentaires sur CEMb lignée PS ont montré que la présence de *L. lactis* V7 modifie l'expression des gènes des interleukines IL-6 et IL-8, des récepteurs *Toll-like* TLR- 2 et TLR-4, ainsi que l'expression du gène de TNF-α dans une culture cellulaire PS infectée avec *E. coli*. L'expression des gènes des interleukines et de TNF-α a aussi été stimulée par la présence de *L. lactis* V7. L'expression de TLR2 et TLR4 a varié selon la souche d'*E. coli* impliquée dans la stimulation. La souche *L. lactis* V7 a donc la capacité de modifier la réponse immunitaire des CEMb pour stimuler l'expression des cytokines responsables de l'inflammation. Il est à noter que ces mesures d'expression ont été menées 24 heures après la stimulation, soit un temps assez tardif par rapport à cette stimulation. Des expériences complémentaires, en cinétique, à différents temps de stimulation, sont cependant nécessaires pour évaluer au mieux l'effet de *L. lactis* V7.

En conclusion, la souche *L. lactis* V7, une BL présente naturellement dans le microbiote de la glande mammaire bovine, est capable d'inhiber l'invasion des cellules de l'hôte *in vitro*

par des pathogènes très importants chez les bovins. Elle stimule également la réponse immunitaire les cellules hôtes. Cette souche présente donc des propriétés intéressantes en tant que probiotique mammaire pour le développement d'une stratégie non antibiotique de lutte contre les mammites bovines.

Pro-inflammatory *Lactococcus lactis* V7 inhibits the invasion of bovine mammary epithelial cells by *E. coli* and *S. aureus*

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Abstract

Bovine mastitis, an inflammatory disease of the mammary gland often associated to bacterial infection, is the first cause of antibiotic use in dairy cattle. Because of the risk of antibioresistance emergence, alternative non antibiotic strategies are needed to prevent or to cure bovine mastitis and reduce the antibiotic use in veterinary medicine. In this work, we investigated *Lactococcus lactis* V7, a strain isolated from the mammary gland, as a probiotic option against bovine mastitis. Using bovine mammary epithelial cell (bMEC) culture, and two representative strains for *Escherichia coli* and for *Staphylococcus aureus*, two major mastitis pathogens, we investigated *L. lactis* V7 ability to inhibit adhesion and internalization of these pathogens into bMEC. *L. lactis* V7 ability to modulate the production of IL-8, a cytokine involved in the inflammatory response, in bMEC upon challenge with *E. coli* was investigated by an ELISA dosage of IL-8 in bMEC culture supernatants. We showed that *L. lactis* V7 inhibited the internalization of both *E. coli* and *S. aureus* strains into bMEC whereas it only inhibited the adhesion of one *S. aureus* strain out of the four strains tested. Investigation of the bMEC immune response showed that *L. lactis* V7 alone induced a slight increase in IL-8 production in bMEC and that it increased the inflammatory response in bMEC challenged with the *E. coli* strains. *L. lactis* V7 thus inhibited the internalization of two major mastitis pathogens into bMEC, induced a slight inflammatory response alone and increased the inflammatory response of bMEC upon challenge with *E. coli*. Altogether these features of *L. lactis* V7 make it a potential promising candidate for a probiotic prevention strategy against bovine mastitis.

Keywords: *Lactococcus lactis, Staphylococcus aureus, Escherichia coli*, mastitis, cell invasion, inflammatory response, mammary probiotics

Introduction

Bovine mastitis is an inflammation of the mammary gland often caused by bacterial infections. *Escherichia coli* (Suojala *et al*., 2013), and *Staphylococcus aureus* (Voelk *et al*., 2014), responsible for environmental mastitis and contagious mastitis, respectively, are major mastitis pathogens in bovine herds. Mastitis causes high economic losses in the dairy farming and industry (Le Maréchal *et al*., 2011). Mastitis associated costs can reach up to US$133 for Gram-positive mastitis and US$211 for Gram-negative mastitis (Cha *et al*., 2011) due to medicines, veterinary charges, milk loss and premature culling. Despite improvements of sanitary conditions during milking, mastitis is far from controlled and it is the first cause of antibiotics use in dairy herd. Antibiotic treatments are not fully efficient, notably against *S. aureus* mastitis, and lead to the presence of antibiotic residues in milk, which may impair the industrial process (Le Maréchal *et al*., 2011). They also contribute to the emergence and transmission of antibiotic resistance within the ecosystems, which include both commensal and opportunistic pathogens. There is thus a need for alternative strategies for mastitis control.

Probiotics appear as an alternative option for mastitis control. Some Lactic Acid Bacteria (LAB) have been studied and used as probiotics in contexts, such as vaginal and intestinal ecosystems, to maintain or restore a microbial balance and to avoid dysbiosis (Ducatelle *et al*., 2014). Although, previous studies revealed the probiotic potential of LAB against bovine mastitis (Klostermann *et al*., 2008), this option remains poorly documented.

We recently reported that *Lactobacillus casei* inhibits the invasion of bovine mammary epithelial cells (bMEC) by *S. aureus* (Bouchard *et al*., 2013). *Lactococcus lactis* was previously shown to inhibit virulence expression in S. aureus *in vitro* and in cheese-

making conditions. In the current study, we used tripartite interactions between bMEC and two major mastitis pathogens to investigate the probiotic potential of *L. lactis* V7, a LAB strain isolated from the bovine mammary gland. Its inhibitory potential against *S. aureus* and *E. coli* invasion into bMEC and its capacity to modulate the bMEC immune response under challenge with *E. coli* was investigated.

Material and methods

Bacterial strains and culture conditions. *Lactococcus lactis* V7 was isolated from swab of a cow teat apex in a Brazilian dairy farm of the Minas Gerais state, in 2011 and identified to species level by 16S DNA sequencing. Four strains of mastitis pathogens were used to assess the probiotic potential of *L. lactis* V7 in terms of invasion (i.e. adhesion and internalization) inhibition and immunomodulation on challenged bMEC. Two well-characterized strains of *S. aureus* isolated from bovine mastitis were used in this study: *S. aureus* RF122 (Herron-Olson *et al*., 2007) and *S. aureus* Newbould 305 (hereafter referred to as N305; Peton *et al*., 2014). *E. coli* strain P4 (Bramley, 1976) and *E. coli* K08, a strain isolated from bovine mastitis in 2003 (available at CIRM-BP, INRA Nouzilly, under reference CIRMBP-548) were kindly provided by Dr. Pierre Germon (UMR ISP, INRA Nouzilly, France).

Subcultures prior to invasion assays were performed overnight as follows: *L. lactis* strain V7 was grown in M17 broth medium (Acumedia, USA) at 30°C without agitation; *S. aureus* strains were grown in Brain Hearth Infusion (BHI, Acumedia, USA) broth medium at 37°C under agitation (180 rpm) and *E. coli* strains were cultured in Luria Bertani broth medium (LB D.Dutscher, Brumath, France) at 37°C under agitation (180 rpm). Prior to invasion assays, subcultures were washed once with PBS and suspended in DMEM **(**D.Dutscher**,** Brumath, France) at different concentrations.

Bacterial concentrations in subcultures were estimated by spectrophotometric measurements at 600 nm with a VWR V-1200 spectrophotometer. One unity of absorbance was equivalent to 7.7×10^8 colony forming units per mL (cfu/mL) for *L. lactis*, 5.7×10^8

and 7.4 x 10^8 cfu/mL for *E. coli* P4 and K08, respectively, and to 1.7 x 10^8 and 2.6 x 10^8 cfu/mL for *S. aureus* RF122 and N305, respectively. They were further confirmed by determination of bacterial population using the micromethod described previously (Baron *et al*., 2006). *S. aureus* population (cfu/mL) was determined on Mannitol Salt Agar (MSA, D.Dutscher) after 24 h incubation at 37°C. *E. coli* population was determined on LB Agar (LBA, D.Dutscher) after 24 h incubation at 37°C. *L. lactis* population was determined on M17 agar (Acumedia) incubated for 24 h at 30°C.

Adhesion and internalization assays

Mammary epithelial cells and culture conditions. The established bovine mammary epithelial cell (MAC-T) line (Nexia Biotechnologies, Quebec, Canada) has been widely used for invasion assays and thus was retained for this study. MAC-T cells were cultured in T75 cell culture flasks in DMEM containing 10 % heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 10 mg/mL streptomycin, and 5 µg/mL insulin (D. Dutscher). Cells were incubated at 37°C in a humidified incubator with 5 % CO_2. They were cultured to a confluent monolayer, treated with 0.05 % trypsin (Gibco-BRL, Grand Island, NY), and suspended in fresh culture medium at a concentration of 2 x 10^5 cells/mL. For adhesion and internalization assays, cells were then seeded in 12-well plates (2 x 10^5 cells/well) and incubated overnight at 37°C in 5 % CO_2 to obtain a confluent monolayer.

Adhesion assay. Adhesion assays were conducted essentially as described previously (Bouchard *et al*., 2013), with slight modifications. Briefly, confluent monolayers of MAC-T cells were prepared in 12 well plates as described above. The interaction was carried out with *L. lactis* V7, *E. coli* (P4 and K08 strains) and *S. aureus* (RF122 and N305 strains) suspended in 1.0 mL of fresh DMEM to achieve a Ratio of Interaction (ROI) of 2000:1 for

L. lactis V7 and a Multiplicity of Infection (MOI) of 100:1 for *E. coli* and *S. aureus*. Bacterial adhesion was measured 1 h post-infection. After washing 4 times the cells on plates with PBS, the monolayer was treated with 0.05 % trypsin, centrifuged for 5 minutes at 800 x g and lysed with 0.01% Triton. The bacterial population that adhered to these cells was determined by colony counting, on M17 agar (Acumedia), Mc Conkey agar (Acumedia) and MSA (D.Dutscher), for *L. lactis*, *E. coli*, and *S. aureus* respectively, from serial decimal dilutions of the lysed cells. Adhesion of the pathogens alone was used as a reference. For adhesion inhibition assay, *L. lactis* V7 and the pathogenic strain were co-incubated for 1 h prior to cell monolayer treatment and bacteria counting.

Internalization assay. Internalization assay was performed following the procedure described previously (Bouchard *et al*., 2013). Confluent monolayers of MAC-T cells, prepared as described above, were washed twice with PBS and incubated with a 1-mL suspension of *E. coli* (P4 or K08) or *S. aureus* (RF122 or N305) and/or *L. lactis* V7 in DMEM at an MOI of 100:1 for *E. coli* and *S. aureus* and an ROI of 2000:1 for *L. lactis*.

Internalization of *L. lactis* V7 alone was measured 2 h post-interaction. After contact, MAC-T cells were washed 4 times with PBS, then incubated for 2 h in 1.0 mL of DMEM supplemented with 100 µg/mL of gentamicin. After this incubation, the cells were lysed and the population of internalized *L. lactis* was determined as described for adhesion assay.

For internalization inhibition assays, *L. lactis* and each of the pathogens were simultaneously added to the cells for 2 h. Pathogens internalization was measured 2 h post-infection following an additional 2-h incubation step with DMEM supplemented with gentamicin (100 µg/mL). MAC-T monolayers were then washed four times with PBS, treated with trypsin, centrifuged for 5 min at 800 x g, and lysed in 0.01% Triton. *E. coli* and

S. aureus populations were determined as described above, for adhesion assays. The internalization assay of *E. coli, S. aureus* alone was used as a reference. Internalization rates were then defined as the internalized *E. coli* or *S. aureus* population in the presence of *L. lactis* relative to the internalized *S. aureus* population in the reference experiment.

Immunomodulation assays. Challenge of PS cells with *E. coli* was shown to induce a pro-inflammatory response (Roussel *et al.*, 2015). To evaluate the pro- and anti-inflammatory effect of *L. lactis* V7 in a mastitis context, the production of IL-8, a cytokine involved in the first step of the inflammatory response, was investigated on PS cell line challenged with *E. coli*.

Mammary epithelial cells and culture conditions. To assess the capacities of *L. lactis* in terms of immunomodulation, we used the newly described bMEC line PS (Roussel *et al.*, 2015). The MAC-T cell line indeed hardly modifies its interleukin genes expression pattern in reaction to contact with pathogens or PAMPs whereas the PS cell line was shown to significantly react to stimulation (Roussel *et al.*, 2015). PS cell line (INRA, Tours, France) was cultured with DMEM – F12 advanced (D. Dutcher) containing 10 mg/mL of IGF-1 (Peprotech), 5 ng/mL of FGF (Peprotech), 5 ng/mL of EGF (Sigma-Aldrich), 1µg/mL of hydrocortisone (Sigma-Aldrich), 20 mM of Hepes buffer (D. Dutcher), and 2 mM of glutamine (Gibco). Plates were incubated at 37ºC in humidified chamber with 5% of CO_2. After trypsin treatment, PS cells were counted in Malassez chamber, and 2.5 x 10^5 cells were transferred into a 24-well plate and incubated for 72 h at 37ºC.

IL-8 determination by ELISA. Stimulation of PS cells with *L. lactis*, *E. coli*, or both species and subsequent measurement of IL8 production were performed essentially as described in Roussel *et al*. (2015). PS cells were seeded at 10^5 cells/well in a 24-well plate and incubated for 72 h at 37ºC (until cells formed a confluent layer). Cells were then cultured overnight (16 hours) in fresh stimulating medium without growth factors (PS stimulation medium). Cells were then washed twice with HBSS (Hank's Balanced Salt Solution; Sigma-Aldrich, Saint-Quentin Fallavier, France) and stimulated with either *L. lactis* V7 at a ROI of 100:1 or *E. coli* P4 or K08 at a MOI of 10:1 or both species. Cells were incubated for 3 h, washed with HBSS and incubated in PS stimulation medium supplemented with 10 µg/mL of gentamicin, for 21 h, completing 24 h of infection. After this incubation, the supernatant was collected and stocked at -20ºC until use. Concentration of IL-8 in the supernatant was measured by ELISA as previously described (Rainard *et al*., 2008).

Statistical analysis

All experiments were performed in triplicate. Statistical analysis was done using the R software (R Development Core Team, 2007). Data were analyzed using the Student t test.

Results

***L. lactis* V7 showed adhesion and a low internalization into MAC-T cells.** Adhesion and internalization tests were carried out to characterize the potential invasion capacities of *L. lactis* V7 strain into bMEC (MAC-T cell line). Experiments with MAC-T cells and *L. lactis* V7 were conducted in a two-step procedure (assay of adhesion and internalization) as described in materials and methods. The results showed that, when *L. lactis* V7 was incubated at a concentration of 2.5 x 10^8 cfu/mL (100%) with MAC-T cells (at ROI 400:1), the number of adhered bacterial cells was of 1.58 x 10^5 cfu/mL (0.0632%), while the number of internalized cells was of only 1.93 x 10^3 cfu/mL (0.0008%).

***L. lactis* V7 inhibited adhesion of *S. aureus* N305 only but inhibited internalization of both *E. coli* and *S. aureus* strains into MAC-T cells.** The inhibitory potential of *L. lactis* V7 on the invasion capacities of *E. coli* or *S. aureus* into MAC-T cells was determined as described in materials and methods. *E. coli* or *S. aureus* were able to internalize into the MAC-T cells within a 2 h period (Figure 1). The conditions used to test the influence of *L. lactis* on the pathogen adhesion were a co-incubation of MAC-T cells with *L. lactis* at ROI = 2000:1 and *E. coli* or *S. aureus* at MOI of 100:1 for 2 h. In these conditions, a significant reduction of the adhesion rate was observed for *S. aureus* N305 only whose adhesion was reduced by 75% (Figure 1A), suggesting that the adhesion inhibition mediated by *L. lactis* V7 is strain-dependent. The effect of *L. lactis* co-incubation upon internalization was also investigated using a MOI of 100:1 for the pathogenic strain and a MOI of 2000:1 for *L. lactis* V7. In these conditions, *L. lactis* V7 reduced the internalization rates of all the *E. coli*

and *S. aureus* strains tested on MAC-T cells (Figure 1B). This inhibition was higher for the *S. aureus* strains (80% and 88% for RF122 and N305, respectively) when compared to the *E. coli* strains (45% and 55% for P4 and K08, respectively), suggesting a wider range of internalization inhibition for *L. lactis* V7 against mastitis pathogens.

***L. lactis* V7 stimulated IL-8 production in PS cell culture challenged with *E. coli* strains.** Production of IL-8, an important interleukin produced during the first steps of inflammation and involved in neutrophils recruitment, was measured to evaluate the effect of *L. lactis* on PS cells infected with *E. coli* P4 and K08 at MOI 10:1. PS cell cultures were first stimulated by the *E. coli* strains and *L. lactis*, alone. PS cells produced higher levels of IL-8 when challenged with both *E. coli* strains (~2000 pg/mL) than in the control condition without any challenge ($p < 0.01$) (Figure 2). *L. lactis* V7 at ROI 100:1 also significantly stimulated IL-8 production by PS cell culture ($p < 0.05$), although at a level (~500 pg/mL) lower than that of *E. coli* challenged cells. The presence of *L. lactis* V7 appeared to highly stimulate the increase of IL-8 production (~3500 and 4000 pg/mL) in PS cell cultures when cells were simultaneously challenged with either of the *E. coli* strains (Figure 2B). Altogether, these results suggest that *L. lactis* V7 induces a slight inflammatory response in PS cells and that co-incubation with *E. coli* and *L. lactis* V7 has a synergistic rather than cumulative effect on IL-8 production.

Discussion

In this study, we investigated *L. lactis* V7, a strains isolated from a cow teat in Brazil, with regard to its probiotic potential in terms of inhibition of pathogens adhesion on and internalization into bMEC by both *E. coli* and *S. aureus* strains, and stimulation of the expression and production of pro-inflammatory cytokines by bMEC.

L. lactis V7 was able to adhere in relatively high amounts to MACT-T cell line (1.58×10^5 cfu/mL, but its internalization was low (1.93×10^3 cfu/mL). The value found for *L. lactis* V7 adhesion is similar to that previously reported for *Lactobacillus casei* strains (Bouchard *et al*., 2013) and other LAB strains (Bouchard D, Seridan B, *et al*. in preparation) onto MAC-T bMEC line. Similar levels were also reported by other authors using different mammary cell lines (Lebeer *et al*., 2007; Vizoso-Pinto *et al*., 2007; Garriga *et al*., 2014). The bacterial capacity to internalize into the host cells is only poorly documented for LAB. Only a few studies report the use of engineered *L. lactis* strains to achieve internalization for intracellular delivery of molecules (Guimaraes *et al*., 2006; Innocentin *et al*., 2009). Here, we showed *L. lactis* V7 was able to internalize into bMEC at a rate comparable to that obtained for another *L. lactis* strain (Bouchard D, Seridan B, *et al*. in preparation). The *L. lactis* V7 internalization rate was however lower than that of other LAB species (Bouchard D, Seridan B, *et al*. in preparation). On one hand, this feature can be considered as an advantage since it considerably limits the risk of tissue invasion by the probiotic candidate. On the other hand, internalization of a probiotic LAB strain might be neutral or even exert a positive effect in the host cell physiology. To our knowledge, this has not been studied as of yet.

L. lactis V7 was able to inhibit the internalization of pathogens frequently associated to mastitis, *E. coli* and *S. aureus* in high percentages ranging from 45 to 88% of inhibition. These values are similar to those previously observed with *L. casei* in bMEC cell lines (Bouchard *et al*., 2013). Interestingly here, the inhibition of internalization did not correlate with the inhibition of the adhesion onto the bMEC, except for *S. aureus* N305, suggesting that different features contribute to these inhibitory potentials. In contrast, adhesion capacities and inhibition of adhesion is a criterion often reported in the selection of LAB candidates against the intracellular pathogen *Listeria monocytogenes* (Nakamura *et al*., 2012; Lavilla-Lerma *et al*., 2013). Altogether, these studies and ours show that the inhibition of a pathogen invasion into its host cell can be mediated by various mechanisms.

A slight production of the pro-inflammatory cytokines IL-8 was observed with *L. lactis* V7 alone and the increase in IL-8 production was greater when PS cells were co-incubated with *L. lactis* V7 and *E. coli* strains. IL-8 is produced in inflammatory conditions, and plays a role in the inflammatory response, and in neutrophils recruitment (Rainard *et al*., 2008). Another *L. lactis* strain (DCP3147) was also shown to enhance the expression of IL-8 (together with IL-6) when injected in a cow teat (Beecher *et al*., 2009). In addition, the intramammary injection of *L. lactis* DCP3147 was able to treat bovine mastitis as efficiently as antibiotics (Klostermann *et al*., 2008). The IL-8 stimulation by *L. lactis* V7 we report here might thus be considered a potential probiotic trait.

In conclusion, we showed here that *L. lactis* V7 was able to inhibit the internalization of *E. coli* and *S. aureus* strains into bMEC. Moreover, *L. lactis* V7 alone was shown to trigger a low inflammatory response on bMEC. When co-incubated with *E. coli*, it significantly amplified the induction of the pro-inflammatory cytokine IL-8

compared the induction obtained with *E. coli* alone. These features could be of interest for the development of a probiotic strategy against mastitis. Inhibition of pathogens internalization can indeed reduce the risk of tissue invasion, infection dissemination and persistence of the pathogens in the mammary gland. In addition, a stronger inflammatory response can enhance the clearance of the pathogen out of the mammary gland. Such property is of particular interest in the case of pathogens inducing a low inflammatory response. These promising results nevertheless require further experiments to be validated in field conditions, e.g. by checking whether or not *L. lactis* V7 induces an increase in somatic cell count when applied in dairy cows and by challenging its protective effect in experimentally induced mastitis.

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Conflict of interest.

The authors declare no financial or commercial conflict of interest.

Figure legends

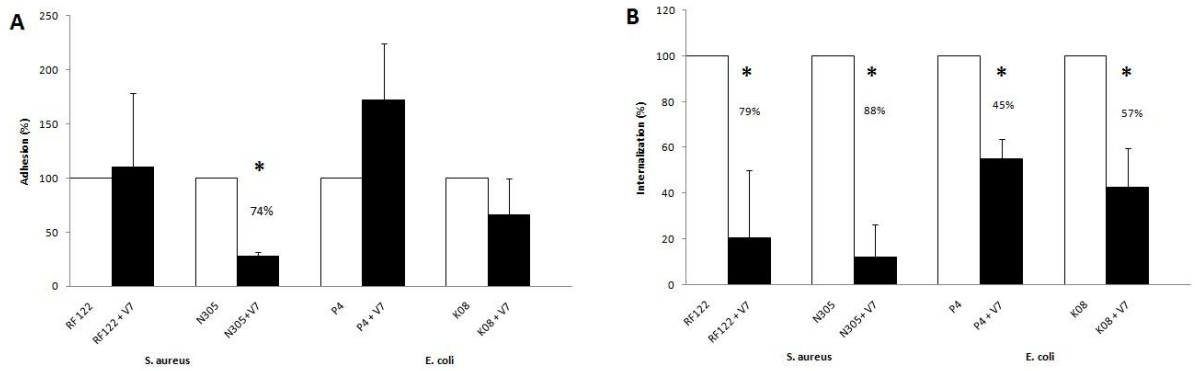

Figure 1. Adhesion and internalization (%) of *E. coli* or *S. aureus* strains into MAC-T cell cultures with or without *L. lactis* V7. (**A)** Adhesion of *E. coli* P4 and K08 and *S. aureus* RF122 and Newbould 305 (N305) was measured after 1h of interaction with MAC-T cells, without (white bars) or with (black bars) co-incubation with *L. lactis* V7. *L. lactis* was used at a MOI of 2000:1, in DMEM and co-incubated with *E. coli* or *S. aureus* challenge at a MOI of 100:1 for 1 h with MAC-T cells. **(B)** Invasion by *E. coli* P4 and K08 and *S. aureus* RF122 and Newbould 305 (N305) strains was measured after 2 h of interaction with MAC-T cells, without (white bars) or with (black bars) co-incubation with *L. lactis* V7. An additional 2h-incubation with gentamicin was carried out before *E. coli* or *S. aureus* counts to kill extracellular bacteria. *L. lactis* was used at a MOI of 2000:1, in DMEM and co-incubated 2 h with MAC-T cells with *E. coli* or *S. aureus* challenge at a MOI of 100:1. Data are presented as mean +/- standard deviation of three independent experiments and differences in relation to the control (pathogen alone) were analyzed using the Student's t test (*) $p < 0.02$.

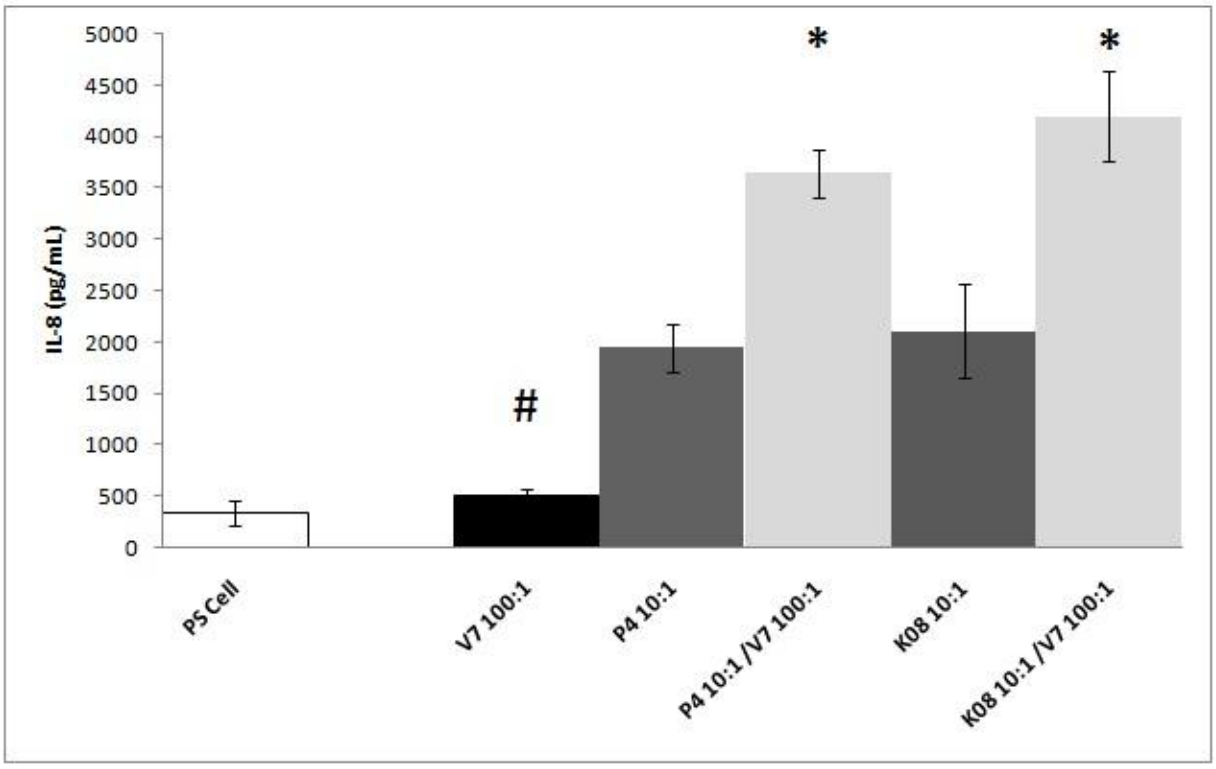

Figure 2. IL-8 production (pg/mL) by PS cell cultures challenged with *E. coli* strains P4 and K08 in the presence or not of *L. lactis* V7. IL-8 production of PS cell cultures alone (PSCell) and in the presence of *L. lactis* V7 (V7, MOI 100:1), *E. coli* P4 (P4, MOI 10:1), *E. coli* K08 (K08, MOI 10:1), a co-culture of *L. lactis* V7 and *E. coli* P4 (V7, MOI = 100:1; P4, MOI = 10:1) and a co-culture of *L. lactis* V7 and *E. coli* K08 (V7, MOI = 100:1; K08, MOI = 10:1). After 24 h of incubation, the supernatant was collected for IL-8 determination by ELISA. Data are presented as mean +/- standard deviation of three independent experiments and differences between groups were analyzed using the Student's t test. (*) $p < 0.03$ when compared to cell with P4 or K08 only; (#) $p < 0.05$ when compared to (PSCell) alone.

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Apêndice

Experiment not presented in this paper

This experiment was not presented in this article becouse it was executed 24 hours after the challenge. Then, in the contrary of the IL-8 detection, the gene expression is not cumulated in the broth and it may be too late to annalyse properly the cell response to *E. Coli* challenge and to *L. Lactis* V7 presence. Thus, as RNA are rapidly degradated, we suggest a future execution of a scinetic of the expression of theses genes to detect the moment of most important expression of each one.

Material and Methods

Challenge of PS cells with *E. coli* was shown to induce a pro-inflammatory response (Roussel *et al.*, 2015). To evaluate the pro- and anti-inflammatory effect of *L. lactis* V7 in a mastitis context, the expression of several genes involved in the innate immune response was investigated on bMEC challenged with *E. coli*.

Mammary epithelial cells and culture conditions

To assess the capacities of *L. lactis* in terms of immunomodulation, we used the newly described bMEC line PS (Roussel *et al.*, 2015). The MAC-T cell line indeed hardly modifies its interleukin genes expression pattern in reaction to contact with pathogens or PAMPs whereas the PS cell line was shown to significantly react to stimulation (Roussel *et al.*, 2015).

PS cell line (INRA, Tours, France) was cultured with DMEM – F12 advanced (D. Dutcher) containing 10 mg/mL of IGF-1 (Peprotech), 5 ng/mL of FGF (Peprotech), 5 ng/mL of EGF (Sigma-Aldrich), 1µg/mL of hydrocortisone (Sigma-Aldrich), 20 mM of Hepes buffer (D. Dutcher), and 2 mM of glutamine (Gibco). Plates were incubated at 37ºC in humidified chamber with 5% of CO_2. After trypsin treatment, PS cells were counted in Malassez chamber, and 2.5 x 10^5 cells were transferred into a 24-well plate and incubated for 72 h at 37ºC.

Determination of cytokine expression

The effect of *L. lactis* V7 on the immune response of PS cells alone or challenged with *E. coli* (P4 and K08 strains) was determined through the evaluation of the expression of genes involved in the immune response by RT-qPCR. This allowed the analysis of several bovine genes for which commercial ELISA kits are not available to assay their gene products. Challenge was performed as previously described (Bonnefont *et al.*, 2011; Porcherie *et al.*, 2012) with some modifications. Briefly, as control, PS cell line was cultured for 72 h and, after this period, the cells were challenged with *E. coli* (P4 or K08 strains) using a MOI of 1:1. To evaluate the effect of *L. lactis* V7 strain on the immune response of challenged PS cells, a co-culture of *E. coli* (MOI 1:1) and *L. lactis* V7 (ROI 100:1) was used. The plates were incubated for 3 h, then the supernatant was discarded, and the cell pellet was washed with PBS and incubated in fresh DMEM supplemented with 10 µg/mL of gentamicin, for 21 h, completing 24 h of infection. After a 24-h incubation, the supernatant was discarded and the cell layer was lysed using lysis buffer from an RNeasy Mini kit (Qiagen) with mercaptoethanol. The lysis product was transferred into microtubes and stored at -20ºC until RNA extraction.

Gene expression analysis

Analysis was performed to determine the expression of the genes encoding IL-6and IL-8, two pro-inflammatory interleukins, TNFα, a cytokine responsible for the apoptosis during inflammation, and TLR2 and TLR4, the Toll Like Receptors responsible for de detection and recognition of pathogens. The expression of these genes can inform on how responsive are the cells submitted to a challenge leading to inflammation. Total RNA was isolated using an RNeasy Mini kit (Qiagen, Courtaboeuf, france). All the extracted RNA was reverse transcribed using a qScript cDNA Synthesis kit (Quanta, France) and pd(N)6 random hexamer (Promega, Madison, USA). Quantitative real-time PCR (qPCR) was carried out using the LightCycler-FastStart DNA MasterPlus SYBR Green I kit on Bio-Rad Chromo 4 Detection System (Bio-Rad, Hercules, USA). Primer pairs (Forward and Reverse) were purchased from Sigma (Angers, France) (Table 1). Samples were normalized using two independent housekeeping genes (GAPDH, RPL19) as controls and reported according to the ΔΔCT method as RNA fold increase: $2^{\Delta\Delta CT} = 2^{(\Delta CT\text{gene of interest} -}$

$^{\Delta CT internal\ control)}$. A relative expression higher than 1 means that the expression is higher in the tested conditions compared to non-infected cells.

Statistical analysis

All experiments were performed in triplicate. Statistical analysis was done using the R software (R Development Core Team, 2007). Data were analyzed using the Student t test.

Results

***L. lactis* V7 modulated the expression of cytokine and Toll-like receptor genes in PS cell line challenged by *E. coli* strains.** We investigated the effect of *L. lactis* on the expression of pro-inflammatory cytokines, and Toll-like receptors genes in PS cells challenged with *E. coli* P4 and K08. The interaction of *L. lactis* V7 alone with PS cells did not trigger a strong induction pro-inflammatory genes as shown by the expression levels of IL-6, IL-8, and TNF-α genes (Figure 2A and 2B). Besides, an increase in the pro-inflammatory cytokine genes expression was observed in PS cells challenged with either of the *E. coli* strains. When PS cells were co-incubated with *L. lactis* V7 and the *E. coli* strains, a significant increase ($p < 0.05$) was observed in the expression of pro-inflammatory cytokines, except for IL-6 gene expression in the P4 challenged group (Figure 1A).

The expression of TLR2 and TLR4 genes increased in PS cells incubated with both *E. coli* strains when compared to PS cells non-infected. Interaction of PS cells with *L. lactis* V7 also induced the expression of TLR2 and TLR4 genes at a roughly similar level compared to the induction by *E. coli* K8 (Figure 1B) whereas it was at a lower level when compared to the induction by *E. coli* P4 (Figure 1A). In PS cells challenged with *E. coli* P4 in the presence of *L. lactis* V7, the expression of TLR2 and TLR4 genes remained at the expression level observed with *L. lactis* alone, resulting in a significant reduction ($p < 0.05$) of TLR gene expression in PS challenged with *E. coli* P4 (Figure 1A) and a slight increase in those challenged with K08 (Figure 1B).

Discussion

L. lactis V7 induced the expression of TLR4 and modulated variably TLR2 compared to the non-infected PS cells. Interestingly, the expression of the two TLR genes was hardly modified when PS cell were co-incubated with *L. lactis* V7 and the *E. coli* strains. *E. coli* P4 alone induced a higher expression of TLR2 and TLR4 when compared to the co-incubation with *L. lactis* V7 or PS cells non-infected, whereas *E. coli* K08 alone induced a lower expression of TLR2 and a faintly higher expression of TLR4 in the same comparation. It thus seems that the effect of *L. lactis* somehow modulate the effect of *E. coli* strains on TLR2 and TLR4 expressions. TLR2 and TLR4 expression in epithelial cells are reportedly the main receptors detecting Gram negative pathogens (Tan *et al*., 2014; Mudaliar *et al*., 2014). They are also involved in the detection of Gram positive probiotic bacteria (Villena *et al*., 2014).

TLR2 gene expression was shown to be upregulated in milk somatic cells in cows suffering from a clinical mastitis (Fonseca *et al*., 2011), and TLR2 and TLR4 gene expression is also upregulated in bovine colonocytes challenged with *E. coli* or its LPS. The effect of *L. lactis* V7 observed here is partially in line with previous findings in other contexts, where "immunobiotic" LAB strains are reportedly capable to reduce TLR2 and TLR4 expression, as well as the subsequent inflammation (Villena *et al*., 2014). The reduction of TLR expression in mastitis has been reported as an anti-inflammatory strategy (Song *et al*., 2014).

These results however contrast indeed with the slight induction of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) observed with *L. lactis* V7 alone and the increase in the expression of these genes when PS cells were co-incubated with *L. lactis* V7 and *E. coli* strains. The induction of IL-8 encoding gene was further confirmed by quantitation of IL-8 production in PS culture supernatants in the same conditions. IL-6 and IL-8 are produced in inflammatory conditions, playing a role in enhancing the immune response, guided by TNF-α expression (Rainard *et al*., 2008). Another *L. lactis* strain (DCP3147) was also shown to enhance the expression of IL-6 and IL-8 when injected in a cow teat (Beecher *et al*., 2009). In addition, the intramammary injection of *L. lactis* DCP3147 was able to treat bovine mastitis as efficiently as antibiotics (Klostermann *et al*., 2008). The IL-6 and IL-8 stimulation by *L. lactis* V7 we report here might thus be considered a potential probiotic

trait. The slight pro-inflammatory effect of *L. lactis* V7 would indeed stimulate the innate immune response and prevent a pathogen invasion. An increased inflammatory response of bMEC, as observed here in the co-incubation of *L. lactis* V7 with *E. coli* strains would enhance the clearance of the pathogens out of the mammary gland. Such property would be of particular interest in the case of pathogens, other than *E. coli*, inducing a low inflammatory response.

Figure Legend

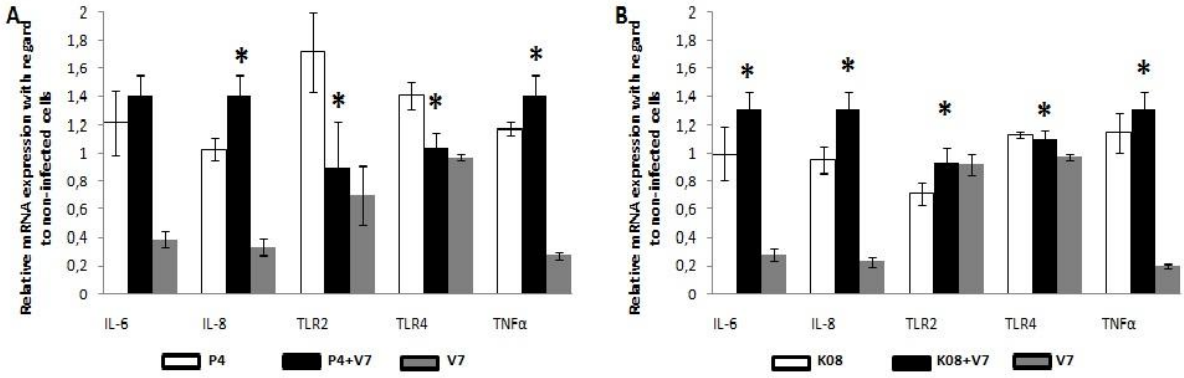

Figure 1. IL-6, IL-8, TLR2, TLR4 and TNF-α gene expression in PS cell cultures challenged with *E. coli* strains P4 and K08 in the presence or not of *L. lactis* V7. (A) Increase of gene expression of PS cell cultures in the presence of *E. coli* P4 (P4, MOI 1:1), *L. lactis* V7 (V7, MOI 10:1) or a co-culture of *E. coli* P4 and *L. lactis* V7 (P4 + V7, MOI 1:1 and 10:1, respectively). **(B)** Increase of gene expression of PS cell cultures in the presence of *E. coli* K08 (K08, MOI 1:1), *L. lactis* V7 (V7, MOI 10:1) or a co-culture of *E. coli* K08 and *L. lactis* V7 (K08 + V7, MOI 1:1 and 10:1, respectively). Data were normalized using housekeeping genes and expressed as relative mRNA expression with regard to PS cell culture alone (negative control). Data are presented as mean +/- standard deviation of three independent experiments and differences in relation to *E. coli* P4 or K08 alone were analyzed using the Student's t test. (*) $p < 0.05$.

Table legends

Table 1: list of the primers used in this study.

Gene Symbol *Bos taurus*	**Oligonucleotides (5'-3') F: Forward; R: Reverse**	**Accession number (Gene Bank)**	**Reference**
IL-6	**F:TGCTGGTCTTCTGGAGTATC R:GTGGCTGGAGTGGTTATTAG**	**EU276071**	Bougarn *et al.*, 2011
IL-8	**F:ACAGAACTTCGATGCCAATGC R:TGTGGCCCACTCTCAATAACTC**	**AF176811**	Fitzgerald *et al*., 2007
TNFα	**F:TCTTCTCAAGCCTCAAGTAACAAGC R: CCATGAGGGCATTGGCATAC**	**EU276079**	Bougarn *et al*., 2011
TLR-2	**F:ACTGGGTGGAGAACCTCATGGTCC R:ATCTTCCGCAGCTTACAGAAGC**	**AF368419**	Goldammer *et al*., 2004
TLR-4	**F: GCATGGAGCTGAATCTCTAC R: CAGGCTAAACTCTGGATAGG**	**DQ839566**	Goldammer *et al*., 2004
GAPDH	**F: GGCATCGTGGAGGGACTTATG R: GCCAGTGAGCTTCCCGTTGAG**	**DQ403066**	Bougarn *et al*., 2011
RPL19	**F:TACTGCCAATGCTCGAATGC R:TGATACATGTGGCGGTCAATC**	**BC102223**	Zimin *et al*., 2009

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Discussão final e perspectivas

Discussão final e perspectivas

Na presente tese foram avaliadas linhagens de bactérias do ácido lático (BAL) a fim de se selecionar candidatas ao uso como probióticos mamários contra a mastite bovina. Para tal, foram isoladas cepas de leite à ordenha, da pele do teto, bem como do canal do teto de bovinos em lactação. Essas cepas foram identificadas por sequenciamento de DNA 16S. Dentre as linhagens confirmadamente pertencentes ao grupo BAL e as espécies reconhecidamente seguras para uso como probiótico, algumas cepas foram selecionadas e caracterizadas quanto a sua interação com patógenos associados à mastite, assim como às células do hospedeiro, em cultivo celular.

A identificação e a caracterização das BAL isoladas do ecossistema mamário bovino, descritas no primeiro capítulo, permitiram o destaque de duas linhagens pertencentes às espécies *Lactobacillus brevis* e uma a *Lactobacillus plantarum*. Esses espécimes foram selecionados em função de sua capacidade de aderir ao epitélio glandular mamário bovino, além de seu perfil pro-inflamatório em ensaios com cultivo celular, características essas que os classificam como bons candidatos ao uso como probióticos mamários bovinos, podendo agir estimulando a resposta inflamatória de defesa fisiológica em casos de mastite infecciosa. Os genomas de cinco dessas linhagens foram sequenciados e a análise genômica (ainda em andamento) permitiu a associação entre certos fenótipos observados *in vitro*, tais como melhor adesão e internalização de certas linhagens à presença ou ausência de alguns genes conhecidos por sua implicação nas interações bactéria-hospedeiro. Outros estudos, tendo como objetivo a avaliação do efeito barreira dessas BAL contra infecções pelos patógenos de maior importância nos casos de mastite bovina, devem ser executados a fim de validar e demonstrar seu potencial probiótico.

No segundo capítulo, foi descrita a seleção de outra linhagem de BAL, mas pertencente à espécie *Lactococcus lactis*. Sua seleção se deveu ao fato de apresentar boa adesão às células hospedeiras bovinas, assim como co-agregar a *Escherichia coli* e por inibir que patógenos associados à mastite invadissem essas células. Dessa forma, a avaliação das

capacidades de adesão a células hospedeiras e sua inter-relação com patógenos e células infectadas por patógenos frequentemente associados à mastite bovina, permitiram concluir que a linhagem lática estudada de *L. lactis* V7 poderia proteger o tecido mamário bovino contra infecções por *E. coli* e *Staphylococcus aureus*.

Em cultura de células mamárias bovinas infectadas por *E. coli*, a mesma linhagem de *L. lactis* V7 estimulou a produção de IL-8. *Lactococcus lactis* V7 ainda aumentou a expressão dos genes das citocinas inflamatórias TNFα, IL-6 e IL-8 e alterou a expressão dos genes dos receptores Toll-like TLR2 e TLR4, que variou com a linhagem de *E. coli* presente na infecção. A avaliação da capacidade de alteração da resposta imune de células hospedeiras infectadas por patógenos frequentemente associados à mastite permitiu concluir que a linhagem de *L. lactis* V7 estimula a resposta imune contra patógenos, devendo atuar na proteção do tecido mamário protegendo-o contra infecções por *E. coli* e *S. aureus* de forma preventiva.

O conjunto dos dados gerados neste trabalho revela que todas as BAL selecionadas foram capazes de promover uma proteção às células mamárias bovinas contra infecções por cepas diferentes de *E. coli* e de *S. aureus.* Contudo, os mecanismos responsáveis por esse fenômeno protetor parecem ser diferentes, envolvendo talvez a competição por sítio de adesão na superfície epitelial e/ou captação do patógeno por co-agregação antes que ele possa aderir-se ao epitélio. A produção de substâncias antagonistas produzidas pelas BAL contra os patógenos poderia ser outro possível mecanismo agindo na superfície epitelial, pois várias das cepas selecionadas apresentaram essa capacidade numa seleção prévia *in vitro* (dados não mostrados). Enfim, a modulação do sistema imunológico do hospedeiro pelas BAL pode ser também considerada. Um aspecto intrigante, encontrado no presente trabalho, foi a internalização das células vivas de BAL nas culturas celulares mamárias sem provocar danos aparentes. Contudo, essa internalização de células bacterianas vivas não patogênicas já foi descrita na mucosa intestinal, o fenômeno de "translocação fisiológica", em que seria responsável pela função de imunomodulação da microbiota normal. Este fenômeno é fundamental para que haja contato entre os componentes dessa microbiota e o sistema imunológico, que se encontram em compartimentos diferentes (Romond *et al*., 2008; Blaser, 2006; Gronlund, 2000).

Este trabalho mostrou também que todas as cepas de BAL foram capazes de desencadear uma resposta imunológica, tanto num modelo infectado como não infectado de cultura celular. Porém, a modulação obtida foi semelhante nos dois capítulos, sendo de caráter pro-inflamatório para as cepas de *Lactobacillus brevis* e *Lactobacillus plantarum* e para a cepa de *Lactococcus lactis* V7. Contudo, essa aparente contradição poderia ser compensada pela dualidade de resultados consequentes de uma inflamação. O processo inflamatório é de fundamental importância para a sobrevida do hospedeiro, para permitir o sucesso de uma resposta imunológica, em particular durante agressões infecciosas, ou "inflamação fisiológica". Contudo, em casos em que resposta inflamatória não é controlada e atinge níveis excessivos, o hospedeiro acaba sendo prejudicado, denominado-se a resposta de "inflamação patológica" (Brandtzaeg *et al.*, 2002). Diversos micro-organismos enteropatogênicos, inclusive *Salmonella* e *E. coli* patogênicas, utilizam este segundo fenômeno, desencadeando sinais pro-inflamatórios nas células epiteliais, após sua adesão, para facilitar a sua invasão posterior (Kalita *et al.*, 2014; Winter *et al.*, 2014). Portanto, uma estimulação pró-inflamatória produzida por uma BAL pode ser benéfica para combater uma infecção, desde que não passe do limiar de fisiológico para o patológico.

Em termos de perspectivas, há, portanto, necessidade de prosseguir com estudos complementares com as cepas selecionadas nos dois capítulos deste trabalho. Os mecanismos de inibição pelas BAL da invasão por diferentes cepas de *E. coli* e *S. aureus* precisam ser mais bem elucidados, pois parece haver fenômenos cepas-específicos, que demandam novos estudos imunológicos, proteômicos e de interação BAL-patógeno em busca dessa resposta. Ainda exigindo esclarecimentos, resta o impacto da modulação da resposta imunológica (anti- ou pró-inflamatória) contra patógenos promovido pelas diversas cepas de BAL selecionadas (*L. brevis, L. plantarum, L. lactis* V7). Isto necessita mais estudos com microscopia, proteômica e ensaios imunológicos, em particular *in vivo*.

Para continuação do presente trabalho de tese, permanece também uma questão quanto à eficácia das cepas selecionadas como alternativa ao uso de antibióticos no tratamento e na prevenção de mastites infecciosas bovinas. Para avançar no possível desenvolvimento de

um produto eficaz ao que se propõe e sua disponibilização no mercado, resta o conhecimento dessa eficácia *in vivo*. Para tal sugere-se o desenvolvimento de estudos, primeiro em modelo murino e, em sequência, ensaio no campo com bovinos. Finalmente, um grande desafio tecnológico, seria o desenvolvimento de uma fórmula de aplicação tópica, onde células de BAL poderiam ser mantidas viáveis e em concentração adequada durante os longos tempos de armazenamento do produto na prateleira de lojas veterinárias.

Discussion finale et perspectives

Dans cette thèse, des souches de bactéries lactiques (BL) ont été évaluées afin de sélectionner des candidates pour une utilisation comme probiotiques contre la mammite bovine. Pour ce faire, des souches ont été isolées à partir de premier jet de lait de traite, de la peau des trayons, ainsi que du canal du trayon de bovins en lactation. Ces souches ont été identifiées par séquençage de l'ADN 16S. Parmi les souches appartenant de fait au groupe des BL et reconnues comme sans danger pour une utilisation probiotique, certaines souches ont été sélectionnées et caractérisées quant à leur interaction avec les agents pathogènes associés à la mammite, ainsi qu´avec les cellules de l´hôte, en culture cellulaire.

L'identification et la caractérisation de BL isolées de l'écosystème mammaire bovin présentées dans le premier chapitre ont permis de révéler des souches appartenant aux espèces *Lactococcus lactis*, *Lactococcus garvieae*, *Lactobacillus brevis*, *Lactobacillus casei* et *Lactobacillus plantarum*. Ces spécimens ont été choisis en fonction de leur capacité d´adhérer aux cellules épithéliales mammaires bovines (CEMb) et à leur profil pro-inflammatoire lors d'essais en culture cellulaire, ce qui les classe comme de bons candidats pour une utilisation comme probiotiques bovins mammaires, pouvant agir en stimulant la réponse inflammatoire de protection en cas de mammite infecctieuse. Les génomes de cinq de ces souches ont été séquencés et l'analyse génomique (encore en cours) a permis d'associer certains phénotypes observés *in vitro* (meilleure adhésion et internalisation de certaines souches) à la présence/absence de certains gènes connus pour leur implication dans les interactions bactéries-hôtes. D'autres études ayant comme objectif l´évaluation de l'effet barrière de ces BL contre l´infection par les pathogènes les plus importants dans le cas de mammite bovine restent à mener pour valider et démontrer leur potentiel probiotique.

Dans le deuxième chapitre, une autre souche de BL a également été sélectionnée, mais appartenant à l´espèce *Lactococcus lactis*. La souche *L. lactis* V7 a été sélectionnée en raison de sa bonne adhésion aux cellules épithéliales mammaires bovines, sa capacité de co-agrégation avec *Escherichia coli* et son inhibition de l´invasion des CEMb par deux

pathogènes majeurs associés à la mammite. Ainsi, *L. lactis* V7 pourrait protéger le tissu mammaire bovin contre les infections par *E. coli* et *Staphylococcus aureus*.

En culture de cellules mammaires bovines infectées par *E. coli*, la même souche de *L. lactis* V7 a stimulé la production d'IL-8. *Lactococcus lactis* V7 a également augmenté l'expression des gènes des cytokines TNF-α, IL-6 et IL-8. L'expression des gènes des récepteurs Toll-like TLR2 et TLR4 a variée quant à elle selon la souche infectante d´*E. coli*. L´évaluation de la capacité de modification de la réponse immunitaire des cellules hôtes infectées par des agents pathogènes souvent associés à la mammite a montré que la souche de *L. lactis* V7 stimule la réponse immunitaire contre les agents pathogènes, pouvant agir ainsi de manière préventive dans la protection des tissus mammaires contre l'infection par *E. coli* et *S. aureus*.

L'ensemble des données générées dans les deux chapitres de cette étude montre que toutes les BL sélectionnées ont été capables d'inhiber l'internalisation des cellules mammaires bovines par différentes souches d´*E. coli* et de *S. aureus*. Cependant, les mécanismes responsables de ce phénomène de protection semblent être différents, impliquant éventuellement la compétition pour site d'adhésion sur la surface épithéliale et/ou l'adsorption de l'agent pathogène sur la BL par co-agrégation avant qu'il n´adhère à l'épithélium. La production de substances antagonistes contre les pathogènes produites par les BL pourrait être un autre mécanisme possible agissant à la surface épithéliale, puisque plusieurs des souches sélectionnées ont montré cette capacité dans une sélection préalable faite *in vitro* (données non présentées). Enfin, la modulation du système immunitaire de l'hôte par les BL pourrait être aussi imaginée. Un aspect curieux observé dans cette étude a été l'internalisation de cellules viables des BL par les CEMb sans dommage apparent pour elles. Toutefois, cette internalisation des cellules bactériennes non pathogènes viables a déja été décrite au niveau de la muqueuse intestinale (translocation physiologique) oú elle serait responsable de la fonction immunomodulatrice du microbiote normal. Ce phénomène est essentiel pour assurer un contact entre les composants du microbiote et le système immunitaire qui sont dans des compartiments différents (Romond *et al*., 2008; Blaser, 2006; Gronlund, 2000).

Cette étude a également montré que toutes les souches de BAL ont été capables de déclencher une réponse immunitaire, aussi bien dans les modèles infectés comme non infectés de culture cellulaire. Cependant, la modulation obtenue a été similaire entre les deux chapitres, avec une nature pro-inflammatoire pour les souches de *L. brevis* et *L. plantarum* et pro-inflammatoire pour la souche *L. lactis* V7. Cette apparente contradiction pourrait être compensée par la dualité des effets qui découlent d´une inflammation. D´un côtê, le processus inflammatoire est crucial pour la survie de l'hôte afin de permettre le succès d'une réponse immunitaire, en particulier lors d´une agression infectieuse, ou «inflammation physiologique». Cependant, si la réponse inflammatoire n´est pas contrôlée et atteint des niveaux excessifs, l'hôte sera affecté, soit l' «inflammation pathologique» (Brandtzaeg *et al.*, 2002). Divers micro-organismes entéropathogènes dont *Salmonella* et *E. coli* pathogénique utilisent d´ailleurs ce deuxième phénomène, en déclenchant des signaux pro-inflammatoires dans les cellules épithéliales après leur adhésion pour faciliter une invasion subséquente (Kalita *et al.*, 2014, Winter *et al.*, 2014). Par conséquent, une stimulation pro-inflammatoire produite par des BL peut être bénéfique pour combattre une infection à condition de ne pas dépasser le seuil physiologique et passer au pathologique.

En termes de perspectives, il est donc nécessaire de poursuivre d'autres études avec les souches sélectionnées dans les deux chapitres de cette thèse. Les mécanismes d'inhibition de l'invasion par différentes souches d´*E. coli* et de *S. aureus* doivent encore être clarifiés, car il semble y avoir des phénomènes spécifiques aux souches, ce qui exigent de nouvelles études immunologiques, de protéomique et d'interaction BAL-pathogène. L´impact de la modulation de la réponse immunitaire (anti ou pro-inflammatoire) contre les agents pathogènes induit par les diverses souches de BL sélectionnées (*L. brevis, L. plantarum, L. lactis* V7) nécessite encore des éclaircissements, ce qui demande d'autres études en microscopie, protéomique et immunologie, en particulier *in vivo*.

Pour la suite de ce travail de thèse, il reste aussi une question quant à l'efficacité réelle des souches sélectionnées comme une alternative aux antibiotiques dans le traitement et la prévention de la mammite bovine infectieuse. Pour favoriser le possible développement

d'un produit efficace qui puisse être mis à disposition sur le marché, il reste à prouver son efficacité *in vivo*. Pour cela, il est nécessaire de mener des essais préliminaires en modèle murin, suivis de test sur le terrain chez les bovins. Enfin, le grand défi technologique, serait le développement d'une formulation topique où les cellules de BL pourraient être maintenues viables et en concentration adéquate pendant la longue durée de stockage d´un magasin de produits vétérinaires.

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