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Implicação das sortases de *Lactobacillus casei* na inibição da internalização de *Staphylococcus aureus* em células epiteliais mamárias bovinas

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ATA DA DEFESA DE TESE

Renata de Faria Silva

Às nove horas do dia **02 de novembro de 2016**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Implicação das sortases de Lactobacillus casei na inibição da internalização de Staphylococcus aureus em células epiteliais mamárias bovinas"**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, o Presidente da Comissão, **Dr. Vasco Ariston de Carvalho Azevedo**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações (O Prof Vasco Azevedo assinou pelo Dr. Michel Gautier – ele participou por vídeo conferência):

Prof./Pesq.	Instituição	CPF	Indicação
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Dra. Sergine Even	INRA	13AY60876	APROVADO

Pelas indicações, a candidata foi considerada: APROVADO

O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 02 de novembro de 2016.

Dr. Vasco Ariston de Carvalho Azevedo - orientador ______

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Dra. Núbia Seyffert - Coorientadora ______

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Dra. Sergine Even - Coorientadora ______

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RESUMO

Staphylococcus aureus é um dos principais agentes causadores da mastite, uma inflamação na glândula mamária. O tratamento da doença baseia-se principalmente em antibióticos. No entanto, os mesmos não são completamente eficazes e seu uso massivo contribui para o risco do surgimento e propagação de linhagens resistentes. Devido a esse risco há uma forte demanda social por uma agricultura mais sustentável, a fim de minimizar o seu impacto sobre o ambiente. Esse fato tem estimulado o desenvolvimento de estratégias alternativas para o tratamento ou prevenção da mastite. Neste contexto, a utilização de bactérias lácticas (BL) como probióticos mamários poderia ser eficaz. As BL são boas candidatas em razão dos efeitos benéficos já descritos em vários contextos. Algumas BL são capazes de interferir com o ciclo infeccioso de agentes patogênicos através de vários mecanismos, entre os quais: inibição direta do crescimento de agentes patogênicos, competição pela colonização de tecidos ou através da modulação da resposta imune inata. Em um estudo anterior, nosso grupo mostrou que a linhagem *Lactobacillus casei* BL23 foi capaz de reduzir a internalização de *S. aureus* em células epiteliais mamárias bovinas (CEMb). No entanto, o mecanismo desse antagonismo ainda premanece desconhecido. As propriedades de superfície de BL23 provavelmente estão implicadas e nós levantamos a hipótese da participação das sortases nessa inibição. Essas enzimas são responsáveis pelo ancoramento das proteínas na parede celular bacteriana. Nós utilizamos quatro mutantes sortases (srtA1, srtA2, srtC1 e srtC2) e um duplo mutante srtA1-srtA2 de BL23 para testar o envolvimento dessas enzimas e seus substratos na inibição da internalização de *S. aureus* em CEMb. Em relação à linhagem BL23 selvagem, todos os mutantes mostraram uma reduzida capacidade de inibir a internalização de *S. aureus* em CEMb. Com destaque para o mutante srtA2, no qual a capacidade inibitória foi completamente abolida. Além disso, experiências preliminares demonstraram a capacidade de BL23 modular a resposta imune em CEMb, com um perfil anti-inflamatório. Os nossos resultados apontam para alguns potenciais mecanismos utilizados por *L. casei* BL23 para exercer os seus efeitos benéficos no contexto da mastite. Esses fatores serão objeto de outros estudos para melhor caracterizar o efeito probiótico exercido por *L. casei* BL23.

Palavras-chave: mastite, *Staphylococcus aureus*, probiótico mamário, sortases.

RÉSUMÉ

Staphylococcus aureus est l'un des principaux agents étiologiques de la mammite, une inflammation de la glande mammaire. Le traitement des mammites à *S. aureus* repose principalement sur l'antibiothérapie. Cependant, les antibiotiques ne sont pas pleinement efficaces et leur utilisation massive contribue au risque d'émergence et de propagation de souches résistantes. A ce risque s'ajoute une forte demande sociétale pour une agriculture plus durable minimisant son impact sur l'environnement. Ceci encourage le développement de stratégies alternatives pour la prévention ou le traitement des mammites. Dans ce cadre, l'utilisation de bactéries lactiques (BL) en tant que probiotiques mammaires pourrait se révéler efficace. Les BL sont en effet de bons candidats en raison des effets bénéfiques déjà décrits dans divers contextes. Certaines BL sont capables d'interférer avec le cycle infectieux d'agents pathogènes par divers mécanismes tels que l'inhibition directe de la croissance des pathogènes, la compétition pour la colonisation des tissus ou la modulation de la réponse immunitaire innée. Lors d'une précédente étude, notre groupe a montré que la souche de *Lactobacillus casei* BL23 était capable de réduire l'internalisation de *S. aureus* dans les cellules épithéliales mammaires bovines (CEMb). Cependant, le mécanisme de cet antagonisme reste inconnu. Les propriétés de surface de BL23 sont probablement impliquées et nous avons émis l'hypothèse de la participation des sortases dans cette inhibition. Ces enzymes sont responsables de l'ancrage des protéines à la paroi des bactéries. Nous avons utilisé quatre mutants sortases (*srtA1*, *srtA2*, *srtC1* et *srtC2*) et un double mutant de BL23 pour tester l'implication de ces enzymes et de leurs substrats dans l'inhibition de l'internalisation de *S. aureus* dans les CEMb. Par rapport à la souche BL23 sauvage, tous les mutants ont montré une capacité d'inhibition de l'internalisation de *S. aureus* au CEMb amoindrie. Pour le mutant *srtA2*, la capacité d'inhibition était totalement abolie. En outre, des expériences préliminaires ont montré la capacité de BL23 à moduler la réponse immunitaire des CEMb, avec un profil anti-inflammatoire Nos résultats pointent certains mécanismes potentiellement utilisés par *L. casei* BL23 pour exercer ses effets bénéfiques dans le contexte de la mammite. Ces facteurs devront faire l'objet d'autres études pour mieux caractériser l'effet probiotique exercé par *L. casei* BL23.

Mots-clés: mammite, *Staphylococcus aureus*, probiotique mammaire, sortases.

ABSTRACT

Staphylococcus aureus is considered one of the main etiological agents of mastitis, an inflammation in bovine mammary gland. The treatment of *S. aureus* mastitis predominantly relies on antibiotics. However, they are not fully effective, and the massive use of antibiotics contributes to the risk of emergence and the spread of antibiotic resistant strains. In addition, there is a strong social demand for a more sustainable agriculture with a lower impact on the environment. This has stimulated the development of alternative strategies for the prevention or treatment of mastitis. This context, lactic acid bacteria (LAB) could be used as mammary probiotics. The beneficial effects of some LAB strains have indeed been demonstrated in various other contexts. They are able to interfere with the infectious cycle of pathogens by various mechanisms, including direct inhibition of pathogen growth, competition for colonization sites of tissues or modulation of the innate immune response. In a previous work, our group showed that *Lactobacillus casei* BL23 was able to reduce the internalization of *S. aureus* in bovine mammary epithelial cells (bMEC). However, the mechanisms underlying this antagonism have not been determined yet. We suspect that surface properties of BL23 are involved in this inhibition capacity and we hypothesized sortases take part to this inhibition. Sortases are responsible for anchoring protein on the bacterial cell wall. We thus used four sortase mutants (*srtA1*, *srtA2*, *srtC1* and *srtC2*) and a double mutant (*srtA1-srtA2*) to test the implication of these enzymes and their substrates in the inhibition of *S. aureus* internalization in bMEC. Compared to the wild type BL23, all the mutants showed an altered inhibition capacity of *L. casei* against *S. aureus* internalization in bMEC. For the *srtA2* mutant, the inhibition capacity was totally abolished. In addition, preliminary results showed that *L. casei* BL23 was able to modulate the innate immune response of bMEC, revealing an anti-inflammatory profile. Our results indicate mechanisms putatively involved in *L. casei* BL23 capacity to exert a beneficial effects in a mammary context. Further studies on these factors are necessary to get a more complete characterization of the probiotic of *L. casei* BL23.

Key-words: mastitis, *Staphylococcus aureus*, mammary probiotic, sortases.

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LISTA DE ABREVIATURAS

Caco-2	= linhagem de adenocarcinoma de cólon humano Caco-2
CD4	= *cluster of differentiation 4*
CD40	= *cluster of differentiation 40*
CD8	= *cluster of differentiation 8*
CFU	= *colony-forming unit*
C-terminal	= carboxi-terminal
CWA	= *cell wall-anchored*
CXCL8	= *chemokine (C-X-C motif) ligand 8*
Da	= Dalton
D-Ala	= D-alanina
D-Asp	= D-aspartato
D-Glu	= D-glutamina
D-Lac	= D-lactato
ELISA	= *enzyme linked immunonosorbent assay*
FOR	= *foward*
g	= gravidade
h	= hora
HT-29	= linhagem de adenocarcinoma de cólon humano HT-29
kV	= quilovolt
L-Ala	= L-alanina
L-Lys	= L-lisina
m/z	= massa/carga
mDAP	= *meso-diaminopimelic acid*
µg	= micrograma
µL	= microlitro
µm	= micrômetro
µM	= micromolar
mg	= miligrama
min	= minuto
mL	= mililitro
mm	= milímetro
mM	= milimolar

nano-LC-MS	= *nanoscale liquid chromatography coupled to tandem mass spectrometry*
NF-κB	= *nuclear factor kappa B*
ng	= nanograma
nm	= nanômetro
NOD	= *nucleotide-binding oligomerization domain*
N-terminal	= amino-terminal
OD	= *optical density*
pH	= potencial hidrogeniônico
ppm	= partícula por milhão
REV	= *reverse*
rpm	= rotação por minuto
s	= segundo
TGF-β	= *transforming growth factor beta*
Th17	= *T helper 17*
TLR	= *toll-like receptors*
TNF-α	= *tumor necrosis factor-alpha*
U	= unidade
UHT	= *ultra-high temperature*
US$	= dólar
v/v	= volume/volume
w/v	= peso/volume
β	= beta
%	= porcentagem
~	= aproximadamente
+/-	= mais ou menos
<	= menor
°C	= grau Celsius
Å	= Ångström

INTRODUÇÃO

1. GENERALIDADES DA PRODUÇÃO LEITEIRA

O leite bovino é um produto de origem animal com elevado valor nutricional e efeitos benéficos para a saúde humana (Micinski et al., 2012). Nas indústrias de lacticínios, essa matéria-prima serve de base para a fabricação de vários produtos, como o leite UHT, creme de leite, iogurte, queijo, manteiga e leite em pó (CNIEL, 2016).

Segundo a Organização das Nações Unidas para Alimentação e Agricultura (FAO), a produção de leite de vaca atingiu em 2012 aproximadamente 625 milhões de toneladas no mundo, com destaque para os Estados Unidos, Índia e China. Por sua vez, o Brasil alcançou uma produção de 32 milhões de toneladas, ocupando a quarta colocação. Enquanto, a França teve uma produção próxima de 24 milhões de toneladas.

O leite possui uma grande importância econômica. Na França, o preço do leite alcançou 309 euros a cada mil litros gerando um saldo comercial de 3,7 bilhões de euros (CNIEL, 2016). No Brasil, esse mercado movimenta anualmente cerca de 64 bilhões de reais, empregando quatro milhões de pessoas (Banco do Brasil, 2010).

A seleção e o melhoramento genético nos rebanhos com o intuito de obter uma maior produção de leite têm um impacto direto sobre a saúde e o bem-estar do animal. Nessa condição, existe uma correlação entre a produção de leite e a incidência de doenças, como a mastite (Oltenacu e Broom, 2010).

2. GLÂNDULA MAMÁRIA

O úbere (Figura 1) possui quatro glândulas mamárias denominadas quartos mamários, que representam as unidades independentes de produção e secreção do leite (Souza, 2015). O desenvolvimento da glândula mamária começa no início da vida fetal em todas as espécies de mamíferos (Ellis et al., 2012).

A glândula mamária é uma glândula exócrina, destinada à secreção de leite (Figura 2). É constituída por células epiteliais que se organizam em estruturas denominadas alvéolos (Cunningham, 2004; Andrews et al., 2008). Cada alvéolo possui um lúmen central (Smith, 2009). Os alvéolos adjacentes formam grupos, os lóbulos, que por sua vez formam estruturas maiores, os lobos. Essas estruturas estão envoltas e ligadas por tecido conjuntivo (Cunningham, 2004). Uma fração do leite recém-sintetizada é drenada dos alvéolos por intermédio de ductos até alcançar as cisternas do úbere e ser armazenada. A outra fração permanece armazenada nos alvéolos até a sua ejeção (Smith, 2009; Coutinho, 2014).

No animal adulto, o ciclo da lactação pode ser dividido em quatro etapas consecutivas, entre elas: a mamogênese, lactogênese, galactopoiese e involução. O primeiro estágio, mamogênese, envolve a proliferação do epitélio celular mamário ao longo da gestação por influência hormonal (Svennersten-Sjaunja e Olsson, 2005). Conforme Trott e colaboradores (2012), a lactogênese é um processo de diferenciação em dois estágios que ocorrem a partir do pré-parto até o parto. Durante o primeiro estágio, ocorre a preparação do epitélio para a síntese dos componentes do leite, o qual irá ocorrer efetivamente no segundo estágio com o estabelecimento da síntese da lactose e a secreção da gordura do leite. A galactopoiese se refere à manutenção da lactação com a remoção regular do leite (Wall e McFadden, 2012). E o intervalo entre o final até o início de uma próxima lactação é um processo complexo envolvendo a involução mamária (Knight et al., 1994). A involução é descrita como a regressão gradual da glândula mamária após completar sua função durante a lactação (Svennersten-Sjaunja e Olsson, 2005).

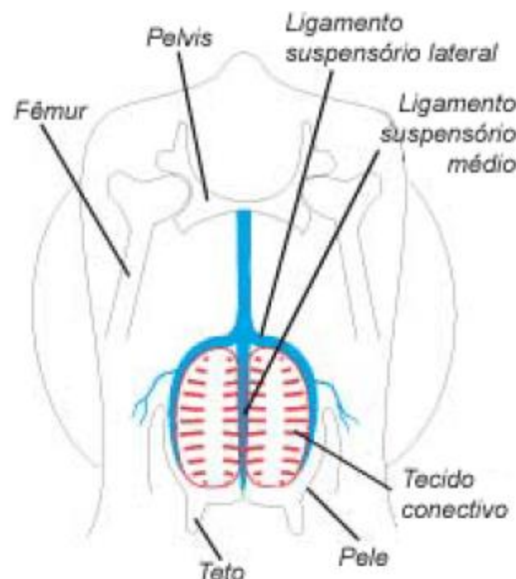

Figura 1. A estrutura do úbere. Adaptado de Sandholm et al., 1990.

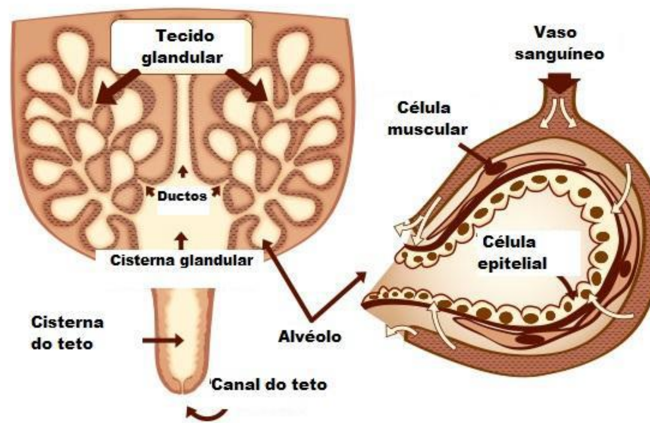

Figura 2. A estrutura da glândula mamária. Fonte: Coutinho, 2014.

2.1. Defesas da glândula mamária

O sistema imune atua na glândula mamária como uma rede de cooperação composta por uma variedade de mecanismos de defesa que podem ser classificados em duas categorias: a imunidade inata e adquirida (Sordillo et al., 1997). A imunidade inata atua predominantemente no estágio inicial da infecção por intermédio de uma barreira física do teto, macrófagos e alguns fatores solúveis. Enquanto, a imunidade adquirida é capaz de reconhecer determinantes antigênicos específicos do patógeno e combatê-los através de macrófagos e anticorpos (Sordillo and Streicger, 2002; Oviedo-Boyso et al., 2007).

O canal do teto da glândula mamária e tecidos associados são considerados como a primeira linha de defesa contra a invasão de bactérias. Uma das barreiras físicas capazes de proteger a glândula mamária da entrada de patógenos é o músculo esfíncter, o qual mantém o orifício do canal do teto fechado propiciando o isolamento do seu interior. Portanto, um dano nessa estrutura pode contribuir para a colonização por organismos causadores da mastite (Myllys et al., 1994; Oviedo-Boyso et al., 2007). Adicionalmente, a camada de queratina que reveste o canal do teto desempenha um importante papel na prevenção da migração de micro-organismos pela cisterna da glândula mamária. Outros fatores associados à queratina como os ácidos graxos, entre os quais ácidos linoleico, palmitoleico e mirístico, funcionam como bacteriostáticos (Capuco et al., 1994; Sordillo e Streicher, 2002; Oviedo-Boyso et al., 2007). Assim como, algumas proteínas catiônicas que ao se ligarem eletrostaticamente aos micro-organismos, modificam sua parede celular aumentando assim, sua sensibilidade às variações osmóticas (Miller et al., 1992; Paulrud, 2005; Oviedo-Boyso et al., 2007).

Caso a primeira linha de defesa da glândula mamária não seja capaz de impedir a invasão dos agentes causadores da mastite. O sistema imune da glândula mamária assegura uma segunda linha de defesa que conta com a ação de macrófagos, neutrófilos e linfócitos. Essas células de defesa participam tanto da imunidade inata quanto da resposta imune adquirida (Sordillo et al., 1997; Soltys e Quinn, 1999; Sordillo e Streicher, 2002; Oviedo-Boyso et al., 2007). No momento em que um micro-organismo é capaz de penetrar no canal do teto, os macrófagos reconhecem o patógeno invasor e inciam a resposta inflamatória. Durante essa resposta pró-inflamatória, algumas citocinas tais como TNF-α e IL-1β, recrutam e estimulam a atividade bactericida dos neutrófilos (Dego et al., 2002; Boulanger et al., 2003; Stein et al., 2003; Oviedo-Boyso et al., 2007). Além do papel dos macrófagos nesse processo, a adesão do micro-organismo nas células epiteliais da glândula mamária, assim como, a interação com toxinas bacterianas induzem a síntese de TNF-α, IL-6 e IL-8 (Rainard e Riollet, 2006; Oviedo-Boyso et al., 2007). Os neutrófilos recrutados para o sítio de inflamação fagocitam o micro-organismo e produzem espécies reativas de oxigênio, peptídeos de baixo peso molecular e defensinas (Sordillo e Streicher, 2002; Mehrzad et al., 2002; Oviedo-Boyso et al., 2007). Finalmente, aqueles patógenos que sobrevivem à atividade dos neutrófilos são combatidos por linfócitos T e B (Rainard e Riollet, 2006; Oviedo-Boyso et al., 2007).

3. MASTITE

A mastite é uma inflamação na glândula mamária causada principalmente por infecções bacterianas. Em menor escala, essa inflamação também pode ser resultante de infecções de origem viral ou fúngica. Além disso, modificações fisiológicas desencadeadas por trauma ou lesão podem contribuir para o desenvolvimento do processo inflamatório (Oviedo-Boyso et al., 2007).

A susceptibilidade do hospedeiro às infecções intramamárias pode ser devido à paridade, nutrição, ao estágio de lactação ou a raça (Oviedo-Boyso et al., 2007). Os mecanismos de defesas do hospedeiro podem ser afetados durante o período de gestação e lactação devido às mudanças nos perfis hormonal e metabólico (Mallard et al., 1998; Wagter et al., 2000). Assim como, a elevação da pressão no interior da glândula mamária de parturientes devido ao ácumulo de fluidos pode causar a dilatação do canal do úbere, e consequentemente, aumentar o risco de infecção (Viguier et al., 2009).

A mastite pode ser classificada em subclínica ou clínica baseada na existência de sintomas locais ou sistêmicos. A mastite subclínica leva a uma menor produção de leite, além

de ser considerada como fonte para novas infecções no rebanho. Enquanto, a mastite clínica causa maiores custos devido ao descarte de leite e tratamento (Lam et al., 2009). Segundo o *National Mastitis Council* (NMC/2006), a mastite clínica é caracterizada pelo aumento no número de células somáticas, assim como, por modificações visíveis na aparência e na qualidade do leite, entre as quais podem ser citadas a formação de flocos e coágulos. No úbere pode ser observado sensibilidade ao toque, inchaço e calor (Bradley, 2002; Oviedo-Boyso et al., 2007). Essa variabilidade pode ser observada até dentro de um mesmo rebanho, no qual é possível identificar animais sadios ou animais com apenas um quarto mamário infectado, ou afetando todos os quartos mamários (Barkema et al., 1997; Le Maréchal et al., 2011). O motivo pelos quais alguns animais desenvolvem mastite subclínica, enquanto outros evoluem para os vários tipos de manifestações clínicas ainda é desconhecido (Vautor et al., 2009). Diante disso, o diagnóstico precoce é de extrema importância.

Atualmente, a contagem de células somáticas (CCS) e a cultura bacteriológica (CB) são os testes diagnósticos mais frequentemente utilizados para a confirmação da mastite. A contagem de células somáticas para avaliar a saúde do rebanho e determinar a qualidade do leite produzido é o método mais amplamente aceito em países que possuem uma indústria leiteira desenvolvida (Rysanek et al., 2007, Brito, 2013). As células somáticas encontradas no leite bovino da glândula mamária não infectada incluem: neutrófilos (1-11%), macrófagos (66-88%), linfócitos (10-27%) e células epiteliais (0-7%) (Lee et al., 1980). Na Europa, CCS acima de 200.000 células/mL é considerada um indicador de mastite (Viguier et al., 2009). A contagem de CCS pode ser feita com amostras de leite provenientes de quartos mamários individuais, produção total diária do animal ou leite total do rebanho (Brito, 2013). Um dos testes mais simples e utilizados é o *California Mastitis Test* (CMT). Um teste de diagnóstico semiquantitativo que avalia a quantidade de células somáticas nas amostras do leite. O reagente do CMT é composto por um detergente (lauril sulfato de sódio a 3%) contendo um indicador de pH (púrpura de bromocresol), o qual é misturado com cerca de 2 mL de leite. Esse detergente é capaz de romper a membrana das células somáticas e reagir com o ácido nucleico, formando uma matriz similar a um gel diretamente proporcional ao número de leucócitos presentes na amostra. A interpretação dos resultados do teste abrange cinco níveis: negativo, reação suspeita ou traços, reação positiva fraca (+), reação positiva (++) ou reação positiva forte (+++) (Viguier et al., 2009; Brito, 2013). As amostras de leite também podem ser avaliadas através da prova de Tamis, popularmente conhecida como teste da caneca de fundo preto, para verificar a presença de grumos e sangue (Souto et al., 2010).

O cultivo bacteriológico, considerado como o teste padrão-ouro para o diagnóstico de infecções intramamárias, pode ser realizado no rebanho ou a nível de quarto. A determinação do *status* de sanidade do animal pode ajudar a prevenir a transmissão da mastite através da identificação da fonte do patógeno no rebanho. Além disso, um conhecimento prévio do histórico de patógenos no rebanho pode ajudar na otimização do tratamento para futuros casos de infecção (Contreras et al., 2006; Lam et al., 2009).

Outros métodos de diagnóticos têm sido desenvolvidos, como a dosagem de enzimas no leite através da dosagem de lactato desidrogenase (LDH) e N-acetil-β-D-glucosaminidase (NAGase), que podem ser detectadas através de ensaios colorimétricos e fluorimétricos (Pemberton et al., 2011; Hiss et al., 2007). O uso do diagnóstico molecular baseado em reação em cadeia da polimerase (PCR, do inglês *Polymerase Chain Reaction*) também tem aumentado nos últimos anos. Apesar de haver a disponibilidade de diagnóstico para vários patógenos causadores da mastite (Lee et al., 1998; Baird et al., 1999, Hassan et al., 2001; Daly et al., 2002), em muitos países o diagnóstico molecular não está disponível como um teste de rotina para a confirmação da mastite (Lam et al., 2009).

3.1. Prevalência e perdas econômicas

A mastite resulta em grandes prejuízos relacionados à redução da produtividade, descarte do leite, gastos com medicamentos, assistência veterinária e perda da qualidade do leite (Halasa et al., 2012). O leite proveniente de animais com a doença apresenta alterações na sua composição química e microbiológica, assim como, em suas características organolépticas (Lopes et al., 2012).

No mundo, estimativas sobre as perdas econômicas devido à mastite clínica indicam um prejuízo de 61 a 97 euros por vaca ao ano (Hogeveen et al., 2011). Na França, a média do custo associada à mastite por *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis* e *Escherichia coli* é estimada em 4.896 euros em um rebanho de 100 vacas leiteiras por ano (Le Maréchal et al., 2011b). No Brasil, o prejuízo identificado referente somente à redução da produção de leite por lactação representa aproximadamente 10% da produção devido à mastite clínica. Enquanto, na mastite subclínica há uma redução da produtividade de 25,5% dos tetos afetados comparado aos sadios (Brito et al., 2007).

3.2. Agentes etiológicos

Os agentes bacterianos causadores da mastite são categorizados em dois grandes grupos: os micro-organismos contagiosos e os ambientais. Cada um desses micro-organismos está implicado aos diferentes graus de severidade apresentados durante o processo inflamatório (Oviedo-Boyso et al., 2007).

Os micro-organismos contagiosos são bactérias patogênicas que vivem e se multiplicam na pele, glândula mamária e podem se espalhar de um quarto mamário para outro em um mesmo animal ou de um animal infectado para um sadio em um mesmo rebanho. Esse grupo inclui: *S. aureus*, *S. agalactiae* e alguns micoplasmas (Bidaud et al., 2007; Bradley et al., 2007). Por outro lado, os micro-organismos ambientais são bactérias patogênicas que estão presentes no ambiente ou utensílios utilizados durante o trato do animal, e nessas circunstâncias podem facilitar a contaminação do teto. Como principais representantes desse grupo podem ser mencionados: *Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Klebisiella pneumoniae* (Bradley et al., 2002).

Na França, um estudo sobre a distribuição dos patógenos implicados na mastite identificou *S. uberis* (22,1%), *E. coli* (16%) e *Staphylococcus* coagulase positiva (15,8%) como os principais agentes causadores da mastite clínica. Enquanto, *Staphylococcus* coagulase positiva (30,2%), *Staphylococcus* coagulase negativa (13,7%) e *S. dysgalactiae* (9,3%) como os patógenos implicados na mastite subclínica (Botrel et al., 2010).

Em 2010, Martins e colaboradores determinaram que 85,2% dos animais possuíam pelo menos um quarto mamário afetado. A mastite clínica atingiu cerca de 5,8%, enquanto a mastite subclínica correspondeu a 65% dos casos, com *Corynebcaterium* sp. (27,6%) e *S. aureus* (21,5%) como os principais agentes implicados no desenvolvimento da doença.

3.3. *Staphylococcus aureus*

3.3.1. Aspectos microbiológicos e bioquímicos

O gênero *Staphylococcus* foi proposto em 1884 por Rosenbach e atualmente inclui 49 espécies e 26 subespécies (Schleifer and Bell, 2010; Euzéby, 2014). Esse gênero é constituído por cocos Gram-positivos com conteúdo guanina-citosina (G+C) entre 30-39%. São bactérias imóveis, não formadoras de esporos e anaeróbios facultativos, podendo ser encontradas de forma isolada ou em grupos, com diâmetro variando entre 0,5 a 1,5 µm. Os membros desse

gênero são positivos para catalase e oxidase-negativos, tolerantes a altas concentrações de sal, além de apresentarem resistência ao calor (Harris et al., 2002).

S. aureus é o membro de maior interesse médico-veterinário, sendo considerado o agente causador de uma ampla gama de doenças humanas (Gordon e Lowy, 2008; Peton e Le Loir, 2014), incluindo septicemia, endocardite, pneumonia, osteomielite, artrite séptica, bacteremia e infecções na pele (Feil et al., 2003; Ishii, 2006). Esse patógeno possui bom crescimento em meios usuais de laboratório, como o *Brain Heart Infusion* (BHI), após 18 à 24 h de incubação a 37 °C sob agitação. As colônias têm um diâmetro aproximado de 1 a 3 mm, com aspecto arredondado, liso, opaco e as vezes com coloração amarelo-alaranjada.

3.3.2. Fatores de virulência

Devido à importância de *S. aureus* no contexto médico-veterinário, vários esforços têm sido aplicados para uma melhor compreensão da estrutura e função dos fatores de virulência, principalmente durante a interação patógeno-hospedeiro (Hecker et al., 2010). O processo de colonização por *S. aureus* é complexo e apenas parcialmente elucidado, principalmente ao que se refere às etapas de contato inicial com o hospedeiro e evasão da resposta do sistema imune (Gordon e Lowy, 2008). Portanto, uma melhor compreensão dos fatores de virulência se faz necessária para evidenciar as diferenças entre as linhagens envolvidas nos diferentes quadros clínicos da mastite (Vautor et al., 2009). Os fatores de virulência são definidos como produtos secretados, componentes estruturais e mecanismos que podem contribuir para a sobrevivência da batéria no hospedeiro (Dego et al., 2002).

Uma classe de fatores importantes durante o processo de estabelecimento de uma infecção são as moléculas adesivas da matriz reconhecedora de componentes de superfície (MSCRAMMs, do inglês *microbial surface components recognizing adhesive matrix molecules*). Esses MSCRAMMs ligam-se às moléculas como colágeno, fibronectina e fibrinogênio (Gordon e Lowy, 2008) e podem mediar a internalização em células eucarióticas (Heilmann, 2011).

As proteínas de ligação à fibronectina A e B (FnBPA e FnBPB, do inglês *fibronectin binding protein*) são um exemplo de fatores que possibilitam a adesão aos tecidos do hospedeiro (Que et al., 2001). FnBPA e FnBPB aderem aos componentes da matrix extracelular, como fibronectina e elastina (Baumstummler et al., 2014). O papel das FnBPs na colonização da glândula mamária também já foi demonstrado com o estudo de mutantes

incapazes de expressar essas adesinas, os quais tiveram a capacidade de adesão às células eucarióticas reduzida em 40% (Dziewanowska et al., 1999).

Vários outros fatores de virulência que contribuem para quadros mais severos da mastite são descritos na literatura, entre os quais leucocidina de Paton-Valentine, superantígenos, toxina alfa, LukM-F' (Le Maréchal et al., 2011; Peton e Le Loir, 2014).

3.3.3. Patogênese

Apesar do processo de patogênese da mastite não ser completamente elucidado, acredita-se que o mesmo ocorra em três etapas sucessivas: invasão, evasão do sistema imune e estabelecimento da inflamação (Oviedo-Boyso et al., 2007). A principal forma de propagação dos micro-organismos é resultante do contato entre animais infectados e sadios. O processo se inicia pela contaminação e invasão da bactéria através do canal do úbere, uma vez que é o único modo de entrada na glândula (Keefe, 2012). Em seguida, a adesão de *S. aureus* nas células do hospedeiro ocorre através de um grupo de adesinas capazes de se ligar à fibronectina, fibrinogênio, colágeno, vitronectina e elastina (Haggar et al., 2003), o que permite sua posterior internalização. A evasão do sistema imune e sobrevivência dentro das células é essencial para *S. aureus* persistir no tecido invadido. Essa habilidade é possível em função da presença de proteínas em *S. aureus* que inibem os efeitos do sistema imune, entre elas: enterotoxinas, protease V8 e estafiloquinase (Jin et al., 2004; Rooijakkers et al., 2005). Por fim, a inflamação é caracterizada pelo aumento de células somáticas e nos casos de mastites clínicas, pelo surgimento dos sinais clínicos próprios da doença (Oviedo-Boyso et al., 2007).

3.4. Métodos de controle da mastite

3.4.1. Abordagens convencionais

Higiene na ordenha. Existe uma relação direta entre o número de bactérias presentes nos tetos e a taxa de infecções intramamárias. Portanto, todos os procedimentos que contribuam para a manuntenção de uma população bacteriana reduzida nos tetos auxiliam no controle da mastite (Santiago et al., 2011). O *pre-dipping* é uma dessas medidas e baseia-se na desinfecção dos tetos com o intuito de reduzir o risco da mastite. Esse processo consiste na imersão dos tetos em solução desinfectante, podendo ser utilizada solução de iodo (0,25%),

solução de clorexidine (de 0,25 a 0,5%) ou ainda cloro (0,2%). A solução desinfectante deve ser aplicada em todos os animais, inclusive naqueles com mastite clínica. (Rosa, 2009).

Antibioticoterapia. No entanto, a principal estratégia para o tratamento da mastite tem sido a antibioticoterapia (Gomes and Henriques, 2016). Vários antimicrobianos têm sido usados para o tratamento da doença, incluindo penicilinas, sulfonamidas, aminoglicosídeos e cefalosporinas (Barkema et al., 2006). Os antibióticos encontrados no mercado apresentam graus variados de eficiência, uma vez que os mesmos não têm a capacidade de penetrar na glândula mamária e portanto, não entram em contato direto com as bactérias. Esse é o caso de animais infectados por *S. aureus* tratados durante a lactação que apresentam um percentual entre 10-30% de eficácia (Bannerman and Wall, 2005). Além disso, a formação de biofilme é considerado como um dos fatores que contribuem para a resistência ao tratamento por antibióticos e portanto, para a recorrência da mastite (Olson et al., 2002). Uma alternativa ao tratamento convencional por antibióticos é a terapia da vaca seca, um método que consiste na infusão lenta de antibióticos na glândula mamária após a última ordenha da lactação. Essa estratégia tem o potencial de eliminar as infecções intramamárias existentes, assim como, prevenir o surgimento de novos casos durante o período seco (Biggs et al., 2016). Apesar da antibioticoterapia ser amplamente adotada para o controle da mastite, seu uso excessivo ou incorreto tem representado um sério risco devido ao surgimento e disseminação de bactérias resistentes para o meio ambiente (White and McDermott, 2001).

Vacinação. Diante disso, a adoção de programa vacinais parece ser uma alternativa mais segura e eficiente ao uso massivo de antibióticos para o tratamento da mastite. Várias abordagens têm sido aplicadas para o desenvolvimento de vacinas com formulações de bactérias atenuadas ou inativadas, extrato total de bactérias ou subunidades bacterianas (Peton e Le Loir, 2014). Algumas vacinas foram desenvolvidas contra *S. aureus*, mas todas apresentaram uma eficácia limitada, seja por esquema inadequado de vacinação ou adoção de adjuvante ineficaz. A proteção limitada da vacina a uma ampla gama de linhagens causadoras da doença também continua sendo um obstáculo, uma vez que várias linhagens podem estar presentes em um mesmo rebanho ou animal (Gomes and Henriques, 2016). Portanto, faz-se necessário uma busca contínua por novos antígenos para o desenvolvimento de vacinas mais eficazes. Vários alvos vacinais têm apresentado potencial para o desenvolvimento de uma vacina, incluindo proteínas de superfície, cápsula de polissacarídeo, biofilme, e toxinas (Scali et al., 2015). Algumas vacinas têm sido testadas sozinhas ou em combinação, entre as quais FnBP-A, CnBP, proteína A, ClfA e cápsula, GAPDH (Peton e Le Loir, 2014). Recentemente, Pujol e colaboradores (2015) avaliaram uma forma trimérica recombinante solúvel do ligante

de CD40 bovino associada a *S. aureus* inativado por calor (HKSA, do inglês *heat-killed Staphylococcus aureus*). Essa formulação foi capaz de estimular a produção de células CD4 (+) especificas para HKSA, assim como, linfócitos T citotóxico CD8 (+) nos nódulos linfáticos de drenagem. Apesar de ser considerada uma alternativa importante e promissora em resposta a patógenos intracelulares, essa estratégia limitou-se ao combate apenas de *S. aureus*.

3.4.2. Abordagens alternativas

Tratamentos alternativos têm focado em medidas naturais com a intenção de reduzir a utilização de substâncias químicas para o controle de diversas doenças em animais (IFOAM, 2005).

Em 2004, um estudo com a utilização de uma substância homeopática foi realizado para avaliar sua eficácia contra a mastite comparando-a ao tratamento com antibiótico. No entanto, nenhuma evidência de eficacidade foi encontrada para essa abordagem (Hektoen et al., 2004). Por outro lado, uma combinação de *Phytolacca*, *Calcarea fluorica*, *Silica*, *Belladonna*, *Bryonia*, *Arnica*, *Conium*, *Ipecacuanha* foi utilizada para tratar 96 quartos com sinais de mastite. Da mesma forma, outros 96 quartos com mastite aguda foram tratados com diferentes antibióticos. Os resultados alcançados foram promissores, com uma efetividade de aproximadamente 86,6% em quartos com mastite sem fibrose e com um custo médio de US$ 0,47. Esses resultados indicam que essa metodologia tem um bom custo-benefício (Varshney e Naresh, 2005). Porém, nenhuma análise foi adotada para identificar os possíveis patógenos causadores da mastite nos animais utlizados nesse estudo. Dessa forma, não é possível atestar a eficiência desse tratamento para uma ampla gama de patógenos. Klocke e colaboradores (2007) avaliaram um tratamento de cinco dias com uma formulação homeopática (*Calcarea carbonica* D30, *Phosphorus* D15, *Pulsatila pratensis* D6, *Atropa Belladonna* D6 e *Lac vaccae* D60) e um tratamento com dose única de *Tuberculinum*. No entanto, essa metodologia não foi capaz de controlar a mastite.

A utilização de óleos devivados de plantas também tem sido considerada como um método alternativo para o tratamento da mastite. Recentemente, um estudo investigou a atividade antimicrobiana do óleo de canola extraído de *Angelica dahurica*, *Angelica sinensis*, *Gaultheria procumbens* e *Glycyrrhiza uralensis* e do óleo de timo contra patógenos causadores da mastite. O óleo de timo foi o único capaz de suprimir o crescimento de *S. uberis* (Mullen et al., 2014). Esse óleo já foi descrito na literatura por possuir atividade

antimicrobiana contra patógenos causadores da mastite (Ananda Baskaran et al., 2009). Além disso, outros óleos essenciais, entre os quais eugenol, carvacrol têm atividade antibacteriana relatada (Burt, 2004).

Várias doenças economicamente importantes em animais, entre elas a mastite, têm sido associadas à deficiência de selênio. Em 2010, um estudo avaliou o efeito de uma injeção de selenito de bário aplicada antes do parto sobre a taxa de incidência de micro-organismos causadores de infecções intramamárias (IMI) e sobre a CCS durante a lactação. No entanto, não houve efeito da suplementação no úbere, o que foi evidenciado através do aumento progressivo de CCS durante a lactação (Ceballos et al., 2010).

Outros estudos para o desenvolvimento de novos antimicrobianos de aplicação veterinária têm ganhado interesse nos últimos anos. O uso de bacteriófagos, vírus capazes de infectar bactérias, tem sido considerado uma ferramenta alternativa à antibioticoterapia para o controle da mastite bovina (Gomes and Herinques, 2016). Várias formulações com bacteriófagos têm sido avaliadas, entre elas a utilização de fago K, MSA6 e peptidase de bacteriófagos. No entanto, observou-se que o fago K foi inibido por secreções do úbere e pelo leite. Isso se torna uma desvantagem, uma vez que o bacteriófago deve estar ativo na glândula mamária (O'Flaherty et al., 2005; Kwiatek et al., 2011; Fenton et al., 2013).

Um dos conceitos emergentes para o controle de diversas doenças se baseia no princípio do controle biológico. A identificação dos vínculos entre a composição de uma determinada microbiota e a saúde do hospedeiro tem despertado o interesse no desenvolvimento de soluções probióticas (Gerritsen et al., 2011; Kamada et al., 2013). A Organização Mundial de Saúde define os probióticos como "organismos vivos que, quando administrados em quantidades adequadas, conferem benefício à saúde do hospedeiro" (FAO/WHO, 2001). Nesse contexto, a fração láctica da microbiota tem sido objeto de estudos e se destacado ao longo dos últimos anos, uma vez que diversas espécies têm demonstrado efeitos probióticos em vários ecossistemas. Diante de sua importância e promissora aplicação no controle de diversas doenças humanas e animais, essa última abordagem será objeto de discussão com mais detalhes no tópico seguinte.

4. POTENCIAL PROBIÓTICO DAS BACTÉRIAS LÁCTICAS NA MICROBIOTA

A microbiota é definida como um conjunto diversificado de micro-organismos, composto principalmente por bactérias, que habitam e desempenham importantes funções em um determinado ecossistema (Zoetendal et al., 2004).

Dentro dessa microbiota é necessário existir um equilíbrio entre as espécies de patógenos oportunistas e comensais não patogênicos, a fim de assegurar a homeostase do organismo hospedeiro (Reid et al., 2011). Esse frágil equilíbrio geralmente se baseia na presença de micro-organismos que exercem um efeito barreira frente a potenciais patógenos (Servin, 2004). A perda desse equilíbrio, devido ao uso de antibiótico, dieta, deficiência imune ou infecção pode contribuir para o surgimento da disbiose (Stecher et al. 2013). Tal condição é definida como uma modificação da composição e/ou abundância das populações microbianas endógenas, com consequente aumento do risco de infecções causadas por espécies patogênicas (Petersen e Round, 2014).

Dentre as diversas espécies que promovem o equilíbrio natural da microbiota, seja através da competição com patógenos pela colonização de tecidos, estímulo da resposta imune ou modulação da expressão de genes implicados na virulência de patógenos, as bactérias lácticas (BL) são as mais bem documentadas (Sengupta et al., 2013; Even et al., 2014).

As bactérias lácticas constituem um grupo numeroso e heterogêneo de micro-organismos conhecidos por sua capacidade em converter açúcar em ácido láctico durante o processo fermentativo (Makarova e Koonin, 2007). Esses micro-organismos são muito utilizados em processos de fermentação de uma variedade de alimentos, tais como: frutas, vegetais, cereais, carne e leite, nos quais contribuem para o sabor, textura e durabilidade (Bron e Kleerebezem, 2011). Uma segunda vertente para o uso de bactéria lácticas é o desenvolvimento de soluções probióticas, entre as quais, as bactérias pertencentes ao gênero *Lactobacillus* sp. são as mais adotadas (Ljungh e Wadstrom, 2006).

Vários estudos têm demonstrado os efeitos benéficos dos probióticos para a saúde humana, apesar da necessidade de comprovação da sua segurança e determinação dos fatores implicados nesses benefícios descritos (Daliri e Lee, 2015). Os principais relatos demonstram a capacidade dos probióticos reduzirem os níveis de colesterol no organismo (Ishimwe et al., 2015), manterem a saúde no ecossistema vaginal através da capacidade de inibição do crescimento de *Candida albincans* (Falagas et al., 2006). Na cavidade oral do homem, tem sido observado que os probióticos podem suprimir espécies causadoras de cáries e reduzir a periodontite (Vivekananda et al., 2010; Reddy et al., 2011). Os efeitos probióticos em suínos, aves e caprinos também têm sido relatados. Em bovinos, o uso de probióticos tem sido explorado quanto a prevenção e combate às desordens digestivas, metabolismo de nutrientes e estímulo de micro-organismos do rúmen que ajudam na promoção da saúde e performance produtiva (Corcionivoschi et al., 2010). No contexto vaginal, foi observado que linhagens de *Lactobacillus* sp. possuem propriedades de superfície diferenciadas e capacidade de inibição

de patógenos conhecidos como causadores de metrites (Otero et al., 2006). Recentemente, essas abordagens também têm sido expandidadas para o contexto mamário bovino.

4.1. Probióticos no contexto mamário

O conhecimento da microbiota em animais tem aumentado, uma vez que há um crescente interesse pela obtenção de uma melhor performance produtiva, nutrição, promoção e manutenção da saúde animal. Com o avanço das técnicas de metagenômica através de sequenciamentos de nova geração, vários esforços estão sendo aplicados na identificação e caracterização da microbiota da glândula mamária bovina (Falentin et al., 2016) e no estudo de formulações probióticas para manutenção da saúde desse ecossistema.

Nesse contexto, um tratamento de mastite subclínica consistindo na utilização de *Lactobacillus acidofillus* e *Lactobacillus casei* foi previamente avaliado como uma abordagem alternativa ao tratamento por antibióticos. Esse tratamento não alcançou bons resultados, uma vez que houve um aumento de CCS com nenhum efeito sobre a taxa de infecção (Greene et al., 1991).

Em 2008, Klostermann e colaboradores avaliaram a eficácia e segurança de uma formulação de *Lactococcus lactis* DPC 3147 para o tratamento de casos subclínicos crônicos e clínicos de mastite bovina ocorridos naturalmente. Essa solução probiótica foi comparada a uma terapia convencional por antibiótico. Os resultados demonstraram que a cultura viva de *L. lactis* tem potencial para ser efetiva na eliminação da doença. No entanto, esse estudo foi apenas uma triagem de campo inicial, tendo assim, a necessidade de uma avaliação de campo mais ampla. Na tentativa de explicar os mecanismos envolvidos nos resultados encontrados, também foi realizado um estudo para verificar o efeito de *L. lactis* na resposta imune. Após a infusão intramamária da formulação probiótica, houve um recrutamento significativo de linfócitos e polimorfonucleares, o que sugere que o mecanismo envolvido é baseado no estímulo da resposta imune do hospedeiro (Crispie et al., 2008). No entanto, sempre deve ser considerado que os probióticos podem exercer seus efeitos como resultado de um ou mais mecanismos combinados (Espeche et al., 2009).

Em 2011, um estudo com a combinação de *Lactobacillus perolens* CRL 1724 e *Lactobacillus plantarum* CRL 1716 foi realizado. Essas bactérias foram previamente isoladas do leite de vaca (Espeche et al., 2009) e tiveram seus potenciais probióticos avaliados através da capacidade *in vitro* de aderir às células epiteliais do canal do teto (BTCEC, do inglês *bovine teat canal epithelial cells*), assim como, pelo potencial inibitório e coagregativo frente

a 14 patógenos conhecidos como agentes causadores da mastite. As avaliações *in vitro* demontraram que *L. perolens* CRL 1724 apresentou um maior potencial probiótico. Portanto, o efeito de uma infusão intramamária com *L. perolens* CRL 1724 em animais com sinais clínicos da mastite foi investigado. Os resultados prelimares demonstraram que a utilização dessa bactéria como um tratamento alternativo não afetou a aparência do leite. Os úberes apresentaram um aspecto normal, no entanto, foi observado um aumento a curto prazo de CCS (Frola et al., 2012).

Vários estudos com o intuito de selecionar bactérias com potenciais probióticos mais promissores também têm sido realizados para o desenvolvimento de formulações probióticas. Uma triagem isolou bactérias lácticas de leite de diferentes rebanhos na Argentina. Cento e dezessete linhagens foram isoladas e caracterizadas quanto à produção de substâncias inibitórias, propriedades de superfície e produção de exopolissacarídeos (EPS). A maioiria dessas bactérias apresentou baixo grau de hidrofobicidade e autoagregação, assim como, nenhuma delas foi capaz de produzir EPS. Contudo, nove linhagens foram capazes de inibir o crescimento de *Listeria monocytogenes* Scott A, *Listeria innocua* 7 e *S. dysgalactiae* através da produção de substâncias inibitórias (Espeche et al., 2012).

Recentemente, nosso grupo de pesquisa isolou bactérias lácticas da microbiota mamária com amostras coletadas do canal do teto. Nesse procedimento, 165 linhagens foram isoladas, dentre as quais, dez linhagens pertencentes aos genêros de *Lactobacillus* e *Lactococcus* foram caracterizadas quanto ao potencial probiótico. Vários critérios foram adotados, entre os quais: hidrofobicidade, autoagregação, potencial inibitório contra patógenos causadores da mastite e propriedades imunomodulatórias. Esse estudo demonstrou que essas linhagens estudadas são candidatas para o desenvolvimento de soluções probióticas apresentando bons resultados, principalmente ao que se refere à atividade anti-inflamatória (Bouchard et al., 2015). Um segundo estudo foi conduzido, no qual foi verificado que *L. lactis* V7 tem a capacidade de inibir a internalização de *S. aureus* e *E. coli* em células epiteliais mamárias bovinas (CEMb), e modular a produção de CXCL8, uma quimiocina responsável pelo recrutamento de neutrófilos, quando desafiados com *E.coli* (Assis et al., 2015).

4.2. Mecanismos de ação

Apesar das soluções probióticas para animais serem amplamente adotadas, os mecanismos subjacentes a seus efeitos benéficos ainda não estão tão bem caracterizados. Contudo, possíveis mecanismos de ação são descritos na literatura, entre eles: habilidade de

adesão e exclusão competitiva de patógenos e modulação da resposta imune (Bermudez-Brito et al., 2012). Durante a exclusão competitiva de patógenos, as bactérias probióticas têm o potencial de reduzir ou impedir o crescimento de micro-organismos patogênicos através da criação de um ambiente hostil, bloqueio físico de receptores bacterianos, secreção de substâncias antimicrobianas e depleção nutricional (Goudarzi et al., 2014).

O processo de competição entre as bactérias probióticas e as bactérias patogênicas pode resultar em um bloqueio dos sítios preferenciais utilizados pelo patógeno para a adesão no tecido hospedeiro (Brown, 2011). Assim como, a comunidade microbiana endógena pode consumir nutrientes preferencialmente necessários para o crescimento dos patógenos desencadeando sua estarvação (Kamada et al., 2013).

A produção de compostos como ácido orgânico ou substâncias denominadas bacteriocinas têm sido propostos por estarem implicados nos efeitos benéficos de probióticos (Bermudez-Brito et al., 2012). Os compostos orgânicos como ácido acético ou ácido láctico têm capacidade inibitória contra patógenos Gram-negativos através da redução do pH intracelular (Alakomi et al., 2000; Makras et al., 2006). Enquanto, a ação bactericida das bacteriocinas é atribuída à capacidade de alterar a permeabilidade da membrana citoplasmática. Várias bacteriocinas já foram identificadas e caracterizadas, entre as quais *nisin*, *diplococcin*, *acidophilin*, *bulgarican*, *helveticins*, *lactacins* and *plantaricins* (Kanaharaj et al., 2012).

As linhagens probióticas também são capazes de estimular a secreção de citocinas a partir de uma variedade de células (células epiteliais, macrófagos e linfócitos) com o potencial de modular a resposta imune inata e adaptativa (Bermudez-Brito et al., 2012). As células do hospedeiro podem reconhecer uma ampla gama de estruturas bacterianas estimulando a secreção de citocinas pró-inflamatórias, como IL-8, em resposta às bactérias patogênicas. E nesse contexto, algumas bactérias probióticas podem estimular uma resposta anti-inflamatória com a finalidade de proporcionar uma homeostase imune e evitar maiores danos celulares (Madsen, 2000).

4.3. Determinantes bacterianos implicados nas propriedades probióticas

4.3.1. Estrutura e função da parede bacteriana

A parede celular possui inúmeras funções durante o crescimento, manutenção da forma e integridade bacteriana. O peptidoglicano (PG) é considerado o principal componente

da parede celular de bactérias Gram-positivas. Ele consiste em cadeias alternadas de N-acetilglucosamina (GlcNAc) e N-acetilmurâmico (MurNAc) unidas através de ligações glicosídicas β 1-4. Embora, o peptidoglicano tenha uma estrutura básica encontrada nas bactérias, ele permanece em estado dinâmico devido aos processos de síntese, maturação e degradação (Chapot-Chartier e Kulakauskas, 2014).

A síntese do PG é basicamente dividida em três etapas: a primeira etapa ocorre no citoplasma com a síntese do lipídeo II. Em seguida, esse lipídeo é transferido para fora da membrana, e por último, ocorre a polimerização dessas unidades recém-sintetizadas (Figura 3) (Typas et al., 2011). O processo de formação do lipídeo II é iniciado a partir da síntese de bifosfato-N-acetilglucosamina (UDP-GlcNAc). Em seguida, UDP-MurNAc é gerado a partir de UDP-GlcNAc através de duas reações enzimáticas catalizadas por MurA e MurB. As ligases MurC, MurD e MurE também participam do processo ao catalizarem à adição de L-Ala, D-Glu e L-Lys ou mDAP, respectivamente. Finalmente, MurF adiciona dois resíduos D-Ala-D-Ala ou D-Ala-D-Lac (Barreteau et al., 2008). O UDP-MurNAc-pentapeptídeo é aderido a um transpotador de lipídeo, o bactoprenol, através da translocase de membrana MraY gerando o lipídeo I. Por último, a ligase MurG adiciona a essa estrutura o GlcNAc formando o lipideo II (Bouhss et al., 2008). Outra modificação do lipídeo II ocorre através de uma reação enzimática como a adição de D-Asp ao aminoácido L-Lys pela ligase aspartato (AslA) (Veiga et al., 2006). Após a síntese completa do lipídeo II, o seu transporte para fora da membrana citoplasmática ocorre por intermédio de uma flipase (FtsW). Por fim, as unidades de lipídeo II são polimerizadas via transpeptidação ou transglicosilação por proteínas de ligação à penicilina (PBPs, do inglês *penicillin*-binding proteins) (Sauvage et al., 2008; Mohammadi et al., 2011; Chapot-Chartier e Kulakauskas, 2014).

No processo de renovação do peptidoglicano, sabe-se da necessidade de um estado de equilíbrio entre os processos de síntese e degradação do peptidoglicano. A perda desse equilíbrio pode resultar em retardo do crescimento e lise bacteriana. A regulação desse importante processo pode se dar a nível transcricional ou pós-transcricional (Chapot-Chartier, 2010; Chapot-Chartier e Kulakauskas, 2014). As hidrolases de peptidoglicano (PGHs, do inglês *peptidoglycan hydrolase*) são enzimas conhecidas por hidrolisarem ligações específicas do peptidoglicano, entre as quais, as autolisinas endógenas que possuem a capacidade de produzir autólise em condições de estresses (Vollmer et al., 2008; Chapot-Chartier, 2010). Em bactérias, diferentes classes de PGHs são encontradas baseadas em sua especificidade de hidrólise. A enzima N-acetilmuraminidase é capaz de hidrolisar a ligação β 1-4 entre MurNAc e GlcNAc, N-acetilglucosaminidase degrada a ligação β 1-4 entre GlcNAc e MurNAc,

enquanto N-acetilmuramil-L-Ala amidase hidrolisa a ligação existente entre MurNAC e o aminoácido L-Ala presente na cadeia lateral peptídica (Chapot-Chartier e Kulakauskas, 2014).

A modificação da parede celular bacteriana através das autolisinas de peptidoglicano tem sido considerada como uma forma das bactérias patogênicas escaparem do sistema imune do hospedeiro (Davis e Weiser, 2011). Os fragmentos de peptidoglicano têm a capacidade de modular a resposta imune do hospedeiro. Esses fragmentos, conhecidos como padrões moleculares associados a micro-organismos (MAMPs, do inglês *microbe-associated molecular patterns*), são reconhecidos por receptores de reconhecimento padrão (PRRs, do inglês *pattern recognition receptors*) presentes na célula do hospedeiro, tais como receptores NOD ou TLR (Wells, 2011). O impacto do peptidoglicano de bactérias probióticas sobre a modulação da resposta imune tem sido pouco relatado (Mokrozub et al., 2015). Por outro lado, esse fenômeno tem sido bem descrito em bactérias patogênicas. O reconhecimento de *S. aureus* extracelular foi determinado como sendo resultante da ligação de fragmentos de peptidoglicano a receptores do tipo Toll 2 (Dziarski e Gupta, 2005), enquanto o reconhecimento intracelular é mais provável ocorrer por intermédio de receptores NOD1 e NOD2 (Travassos et al., 2004).

Na superfície da parede celular bacteriana, as proteínas são encontradas somente depois de atravessarem a membrana citoplasmática e se associarem a algum componente da superfície, como o peptidoglicano. A localização na superfície requer que essas proteínas possuam dois domínios, o primeiro é um motivo sinal localizado na região N-terminal da proteína, que geralmente medeia a sercreção através da via Sec de secreção (Tjalsma et al., 2004). O segundo é um motivo que direciona o ancoramento das proteínas de maneira covalente ou não covalente na superfície celular bacteriana. As proteínas com associação não covalente no envelope celular possuem domínios específicos, entre os quais: módulos GW, domínio WxL, domínio LysM e domínio de ligação ao peptidoglicano (Bierne e Cossart, 2007). Enquanto, algumas proteínas com ancoramento covalente possuem um motivo LPXTG (leucina, prolina, qualquer aminoácido, treonina e glicina) na região C-terminal, seguida por uma região hidrofóbica e uma cauda de aminoácidos carregada positivamente (Figura 4a) (Cascioferro et al., 2015). Em bactérias Gram-positivas, essas proteínas LPXTG são ancoradas covalentemente na parede celular bacteriana através de uma tiol-transpeptidase ligada à membrana, chamada sortase. Essa enzima é capaz de reconhecer o motivo LPXTG entre os resíduos treonina-glicina e catalisar uma ligação entre o grupo carboxil C-terminal do resíduo treonina e um grupo amino do peptidoglicano (Figura 4b) (Muñoz-Provencio et al., 2012).

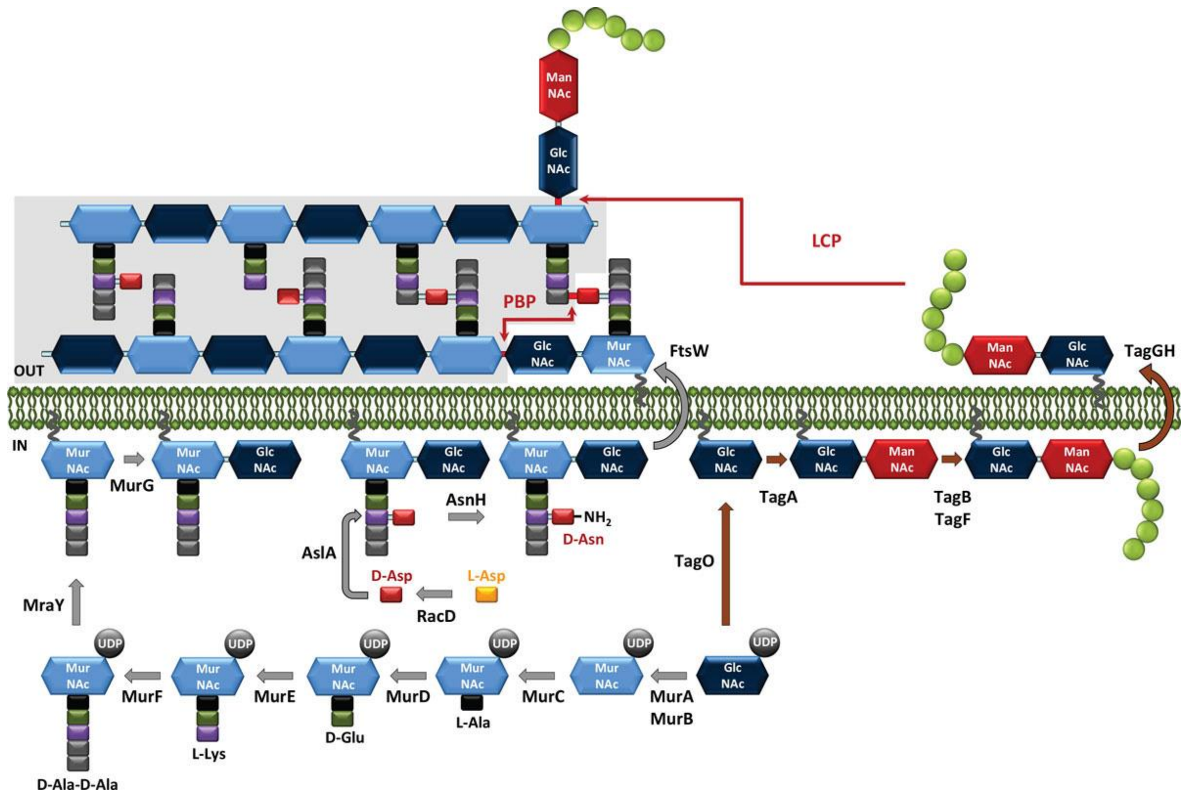

Figura 3. Representação esquemática das principais etapas de síntese do peptidoglicano. Fonte: Chapot-Chartier e Kulakauskas, 2014. As setas cinzas indicam os passos de biossíntese do PG e as setas marrons indicam os passos de biossíntese do ácido teicóico. As ligações formadas por PBP são indicadas com setas vermelhas. O peptidoglicano pré-existente está destacado por um retângulo cinza.

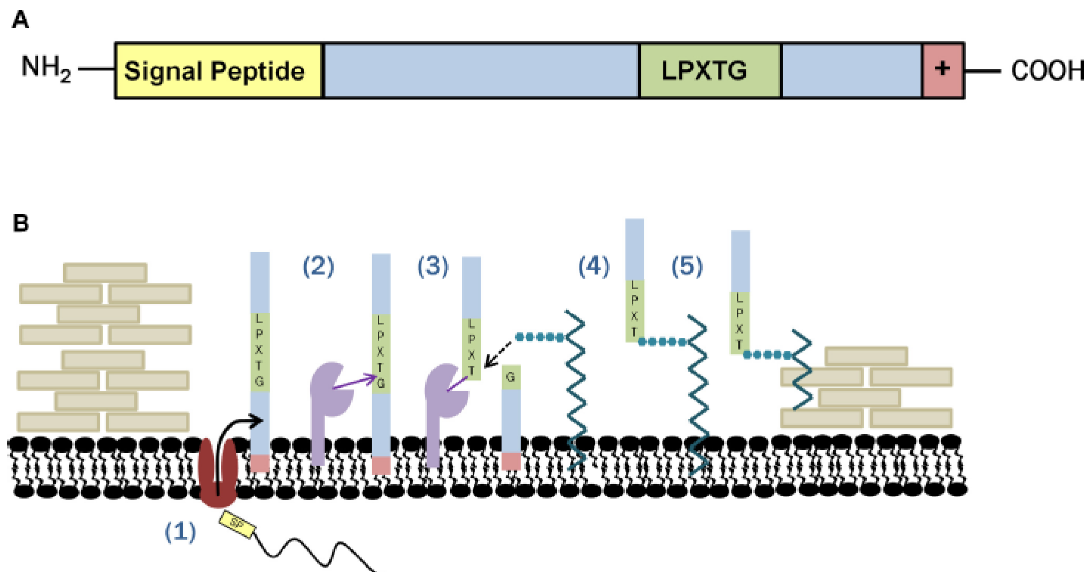

Figura 4. Anconramento de proteínas de superfície com motivo LPXTG no envelope celular de bactérias Gram-positivas através da enzima sortase. Fonte: Call e Klaenhammer, 2013. A) Os substratos sortases são reconhecidos devido à presença de um peptídeo sinal N-terminal e um sinal de reconhecimento LPXTG na região C-terminal, seguido por uma série de resíduos hidrofóbicos e carregados positivamente que facilitam o ancoramento na membrana. (B) A ligação covalente dos substratos sortases à parede celular é realizada através de cinco etapas: (1) A maquinaria Sec reconhece o peptídeo sinal do substrato sortase e exporta o substrato para o exterior da célula. O substrato permanece incorporado na membrana devido à presença de uma região hidrofóbica seguida por uma cauda carregada positivamente. (2) Uma vez que o substrato sortase e a enzima sortase estão próximos, a sortase cliva entre os resíduos glicina e treonina através de uma reação de transpeptidação. (3) A ligação nucleofílica ao lípideo II desassocia o complexo sortase/SDP e (4) forma um lípideo II intermediário através da interação com a ponte cruzada de peptídeos. (5) Nos estágios finais do ancoramento, o substrato sortase é incorporado na parede celular.

4.3.2. Proteínas de superfície de bactérias lácticas com implicação em eventos de adesão e autoagregação bacteriana

Recentemente, vários estudos têm demontrado a influência de componentes da superfície celular de bactérias lácticas sobre a habilidade de adesão e autoagregação bacteriana (Mokrozub et al., 2015). A superfície celular de BL é coberta por ácidos teióicos, proteínas de superfície e polissacarídeos (Sengupta et al. 2013). As proteínas de superfície não são os únicos fatores envolvidos nessas habilidades, no entanto, elas possuem importantes papéis que auxiliam na compreensão dessa complexa interação com o hospedeiro (Li et al., 2015).

Estudos têm sido realizados com o intuito de determinar o papel das proteínas de superfície na interação com o hospedeiro. Nesse contexto, foi demonstrado que a capacidade

de adesão à linhagem celular HT-29 e o perfil autoagregativo de *Lactobacillus rhamnosus* PEN estão associados ao fato dessa linhagem possuir proteínas de superfície específicas e conteúdo de ácido graxo único (Polack-Berecka et al., 2014). Em concordância com esses resultados, uma outra avaliação após à extração das proteínas de supérfície com cloreto de lítio também identificou uma redução da capacidade de adesão e autoagregação em BL (Li et al., 2015). Os dois estudos determinaram a importância das proteínas de superfície durante os processos de adesão e autoagregação, porém não identificaram especificamente quais dessas proteínas poderiam potencialmente estar envolvidas nesses fenômenos. Por outro lado, em *L. acidophilus*, três linhagens mutantes para a proteína de ligação ao muco, proteína de ligação a fibronectina e uma proteína *S-layer* foram obtidas. Os resultados demonstraram uma redução da capacidade de adesão à células Caco-2 de 65%, 76% e 84%, respectivamente. Contudo, o efeito encontrado para o mutante de *S-layer* pode ter sido acumulativo devido à perda de múltiplas proteínas (Buck et al., 2005). Assim como, em 2008, o papel das proteínas inulosucrase (IlsA) e glicosiltransferase (GtfA) de *Lactobacillus reuteri* TMW1.106 também foi investigado. A utilização das linhagens mutantes revelaram que ambas as proteínas contribuem para a habilidade de autoagregação em pH ácido. Além disso, GtfA e IlsA auxiliam na formação de biofilme (Walter et al., 2008).

Em *L. casei* BL23, análises de espectometria de massa revelaram a presença de proteínas de resposta ao estresse (GroEL, CLpL), fatores de elongação de tradução (EF-Tu, EF-G) e enzimas implicadas na via glicolítica como a enolase e gliceraldeído 3-fosfato desidrogenase (GAPDH) na sua superfície. Posteriormente, ensaios *in vitro* com as proteínas enolase e GAPDH demonstraram uma atividade de ligação dessas proteínas à fibronectina e colágeno, reforçando a ideia que *L. casei* BL23 possui inúmeras proteínas *moonlighting* em sua superfície (Muñoz-Provencio et al., 2011). A maioria das proteínas tem uma única função descrita na literatura, mas várias delas podem desempenhar uma função adicional em uma localização celular diferente, sendo designadas como proteínas *moonlighting* (Huberts e van der Klei, 2010). Várias proteínas *moonlighting* exibindo propriedades adesivas têm sido identificadas em espécies de *Lactobacillus*, entre as quais GAPDH, enolase, glucose-6-fosfato isomerase, triose fosfato isomerase, fosfoglicerato isomerase, fator de elongação Tu, glutamina sintetase e GroEL (Kainulainen e Korhonen, 2014).

4.4. *Lactobacillus casei BL23*

4.4.1. Generalidades

O gênero de *Lactobacillus* abrange cerca de 80 espécies que são classificadas como micro-organismos Gram-positivos, não formadores de esporos, anaeróbios aerotolerantes e ácido-tolerantes (Buriti et al., 2007). Dentro desse gênero, *Lactobacillus casei* é uma espécie heterofermentativa facultativa amplamente utilizada nas indústrias de alimentos como cultura iniciadora de processos de fermentação do leite ou maturação de queijos, assim como probióticos (Alcántara e Zúñiga, 2012). São bacilos variando 0,7-1,1 µm de largura por 2,0-4,0 µm de comprimento com colônias geralmente lisas, com bordas irregulares e frequentemente translúcidas (Pereira, 2007). A linhagem BL23 possui um conteúdo G+C de 46,34%, sendo bastante adotada para o desenvolvimento de estudos genéticos, fisiológicos e bioquímicos (Rico et al., 2008; Mazé et al., 2010).

4.4.2. Propriedades probióticas de *L. casei* BL23

O potencial probiótico e a capacidade de modulação da resposta imune de *L. casei* BL23 é normalmente investigada no contexto intestinal humano, exceto por um estudo que avaliou sua contribuição na glândula mamária bovina realizado por Bouchard e colaboradores (2013) que será discutido com mais detalhes adiante.

A avaliação da propriedade preventiva de *L. casei* BL23 em modelo murino de colite moderada induzida por sulfato de sódio dextrano (DSS, do inglês *dextran sulphate sodium*) revelou que a linhagem tem a capacidade de reduzir a inflamação no cólon e ceco (Rochat et al., 2007). O resultado obtido sugere que a catalase dependente de manganês não apresenta contribuição para essa proteção (Rochat et al., 2007), apesar de alguns estudos demonstrarem a influência do acúmulo de espécies reativas de oxigênio em danos epiteliais oxidativos (Lih-Brody et al., 1996; Keshavarzian et al., 2003).

Além disso, um estudo avaliou se a matriz de entrega pode influenciar na capacidade de proteção dessa linhagem probiótica. Para isso, foi utilizado um modelo murino de colite ulcerativa induzido por DSS. Foi possível determinar que *L. casei* BL23 combinado ao leite mantém suas caracteríticas probióticas, entretanto, quando usada em associação a um tampão livre de nutrientes, a bactéria perde seu potencial de proteção. Assim como, linhagens mutantes para DLT (do inglês, *lipoteichoic acid D-alanyl transfer protein*), uma proteína

responsável pela D-alanilação do ácido lipoteicóico previamente associada à modulação da resposta imune em modelo murino de colites e RecA (do inglês, *recombinase A*), uma proteína importante na tolerância ao estresse oxidativo e outros estresses ambientais perdem a capacidade de proteção contra a colite. Após a ingestão de BL23, também foi identificada uma quantidade reduzida de algumas citocinas pró-inflamatórias, tais como IL-6, IL-1α e KC (Lee et al., 2015).

Recentemente, o potencial probiótico de *L. casei* BL23 no tratamento de câncer de cólon retal induzido por 1,2 dimetilhidrazina também foi demonstrado em modelo murino. A resposta imune, células T e produção de citocinas, foi avaliada após a incubação da bactéria com células esplênicas de camundongos não tratados. A proteção de *L. casei* BL23 nessa condição está relacionada à modulação de células T regulatória através da resposta imune Th17 acompanhada da expressão de IL-6, IL-17, IL-10 e TGF-β em esplenócitos tratados com a bactéria e IL-22 *in vivo* (Lenoir et al., 2016).

5. PROBLEMÁTICA DA TESE

Em 2013, nossa equipe de trabalho avaliou a habilidade de três linhagens de *L. casei* em inibir a adesão e internalização de *S. aureus* N305 em CEMb. Entre as linhagens avaliadas nesse estudo, *L. casei* BL23 foi selecionada, uma vez que é uma linhagem bem caracterizada e com genoma sequenciado disponível. Nesse estudo, nós observamos que BL23 tem a capacidade de inibir a internalização de *S. aureus* N305. Experiências preliminares para determinar quais mecanismos poderiam estar implicados nesse antagonismo mostraram que *L. casei* BL23 deve estar vivo para essa inibição ser observada. Além disso, um contato direto entre *L. casei* com CEMb e/ou *S. aureus* é necessário (Bouchard et al., 2013).

O antagonismo de *L. casei* sobre a internalização de *S. aureus* provavelmente envolve um ou mais mecanismos combinados, entre os quais, a modulação da resposta imune do hospedeiro, competição pelos sítios de invasão e a modulação da expressão de genes implicados na virulência de *S. aureus*. Vários estudos têm demontrado a influência de componentes da superfície celular nessas propriedades probióticas. Diante disso, nossa hipótese é que as proteínas de supérfície de *L. casei* BL23 estão implicadas nesse processo de antagonismo sobre a internalização de *S. aureus*. As proteínas de superfície são particularmente interessantes devido ao importante papel que desempenham na interação das bactérias com as células do hospedeiro. No entanto, a avaliação da implicação de cada

proteína presente na superficie de *L. casei* seria muito longa. Uma abordagem alternativa para essa avaliação é o estudo do papel das sortases nesse antagonismo.

As sortases são enzimas implicadas no ancoramento de várias proteínas na parede celular bacteriana. Ao testar a implicação das sortases, nós testamos a implicação de seus substratos, as proteínas CWA (do inglês, *cell wall anchor*). Para comprovar nossa hipótese, a abordagem adotada foi a utilização de linhagens mutantes de *L. casei* BL23, onde um ou vários genes sortases (srt) foram inativados. Nós testamos o impacto desses mutantes sortases na inibição da invasão de *S. aureus* nas células epiteliais mamárias bovinas. Em paralelo, as mudanças no perfil de proteínas ancoradas na superfície bacteriana de *L. casei* BL23 selvagem e dos mutantes foram analisadas de maneira a identificar os potenciais determinantes dessa inibição.

Além da capacidade em inibir a colonização de tecidos por patógenos, os probióticos frequentemente têm a capacidade de interferir com a resposta imune inata. A fim de explorar de maneira mais abrangente o potencial probiótico de *L. casei* BL23 no contexto mamário, nós realizamos o estudo do impacto dessa linhagem sobre a resposta imune inata em células epiteliais bovinas.

Esses trabalhos são apresentados sob a forma de artigos na seção de Resultados desse manuscrito.

INTRODUCTION

1. MAMMITE

Selon l'Organisation des Nations Unies pour l'alimentation et l'agriculture (FAO), la production de lait de vache en 2012 a atteint environ 625 millions de tonnes dans le monde et elle revêt de fait une grande importance pour l'économie du secteur agro-alimentaire mondial. La sélection et l'amélioration génétique dans les troupeaux afin d'obtenir une production de lait plus élevée a entraîné un impact direct sur l'incidence des maladies, telle que la mammite (Oltenacu et Broom, 2010).

La mammite est une inflammation dans la glande mammaire, le plus souvent d'origine infectieuse, qui se traduit par des pertes importantes liées à la réduction de la productivité, la perte de la qualité et de l'élimination du lait, les dépenses en médicaments et les soins vétérinaires (Oviedo-Boyso et al., 2007 ; Halasa, 2012). Actuellement, le comptage de cellules somatiques et la culture bactérienne sont les tests de diagnostic les plus souvent utilisés pour la confirmation de la maladie (Rysanek et al., 2007 ; Contreras et al., 2006 ; Lam et al., 2009 ; Brito, 2013). Différents agents peuvent être impliqués dans le développement de l'infection qui présente divers degrés de sévérité de la mammite (Oviedo-Boyso et al., 2007 ; Bidaud et al., 2007 ; Bradley, 2002) . Ceux-ci incluent *Staphylococcus aureus* qui est une bactérie pathogène responsables de nombreuses infections et qui pose un problème majeur en médecine vétérinaire (Gordon et Lowy, 2008 ; Peton et Le Loir, 2014).

La principale stratégie pour le traitement de la mammite est l'antibiothérapie. Cependant son efficacité n'est pas toujours optimale, notamment sur les mammites à *S. aureus* et elle engendre un risque d'émergence et de propagation de bactéries antibio-résistantes dans l'environnement (White et McDermott, 2001 ; Bannerman et Wall, 2005 ; Gomes et Henriques, 2016). De ce fait, le développement de vaccins avec des formulations de bactéries atténuées ou inactivées, des extraits totaux de bactéries ou de sous-unités bactériennes semble être une alternative plus sûre et plus efficace (Peton et Le Loir, 2014). Certains vaccins ont été développés contre *S. aureus*, mais tous ont une efficacité limitée (Gomes et Henriques, 2016). Les traitements alternatifs sont également considérés, entre lesquels, le traitement homéopathique (Hektoen et al., 2004 ; Varshney et Naresh, 2005 ; Klocke et al., 2007), les huiles essentielles (Mullen et al., 2014), la supplémentation alimentaire (Ceballos et al., 2010) et les bactériophages (O'Flaherty et al., 2005 ; Kwiatek et al., 2011 ; Fenton et al., 2013 ; Gomes et Herinques, 2016).

L'un des nouveaux concepts pour le contrôle de plusieurs maladies est basé sur le principe de la lutte biologique. L'identification des liens entre la composition d'un microbiote

particulier et la santé de l'hôte a suscité un intérêt dans le développement de solutions probiotiques (Gerritsen et al., 2011 ; Kamada et al., 2013). Dans ce contexte, la fraction lactique de divers microbiotes (par exemple, intestinal et vaginal) a été étudiée au cours des dernières années et de nombreuses espèces ont montré des propriétés probiotiques pour leurs hôtes.

2. POTENTIELS PROBIOTIQUE DES BACTÉRIES LACTIQUES

Le microbiote est défini comme une communauté variée de micro-organismes composée principalement de bactéries qui vivent et jouent un rôle important dans un déterminé écosystème (Zoetendal et al., 2004). Dans ce microbiote, il doit y avoir un équilibre entre les espèces d'agents pathogènes opportunistes et les espèces commensales non pathogènes pour assurer l'homéostasie de l'organisme hôte (Reid et al., 2011). Cet équilibre délicat est généralement basé sur la présence de micro-organismes qui exercent un effet barrière vis à vis des agents pathogènes potentiels (Servin, 2004). La perte de cet équilibre en raison de l'utilisation d’antibiotique, de l'alimentation, de la déficience immunitaire ou d’une infection peut contribuer à l'émergence d’une dysbiose (Stecher et al,. 2013). Cette condition est définie comme un changement dans la composition et / ou dans l'abondance des populations microbiennes endogènes, avec un risque augmenté d'infections provoquées par des espèces pathogènes (Petersen et Round, 2014).

Parmi les différentes espèces qui favorisent l'équilibre naturel de certains microbiotes d’intérêt pour l’homme ou l’animal, les bactéries lactiques (BL) sont les mieux documentées (Sengupta et al., 2013 ; Even et al., 2014). Les BL constituent un groupe important et hétérogène de micro-organismes connus pour leur capacité à convertir divers sucres en acide lactique au cours du processus de fermentation lactique (Makarova et Koonin, 2007). A ce titre, elles sont largement utilisées en industrie agroalimentaire. Un second aspect de l'utilisation des BL est le développement de solutions probiotiques dans lesquelles on retrouve couramment des bactéries appartenant au genre *Lactobacillus* sp. (Ljungh et Wadstrom, 2006).

Plusieurs études ont montré des effets bénéfiques de probiotiques pour la santé humaine. Ces études doivent être complétées de travaux montrant la sécurité des souches employées et déterminant les facteurs impliqués dans les avantages décrits (Daliri et Lee, 2015). Récemment, ces approches ont également été étendues pour le contexte mammaire bovin. Notre connaissance et la prise en compte du microbiote chez les animaux de rente a

augmenté ces dernières années car les acteurs des filières considèrent qu'il peut jouer un rôle (comme la génétique ou la nutrition) dans l'obtention d'un meilleur rendement de production et dans la promotion et le maintien de la santé animale. Avec les progrès des techniques de métagénomique par séquençage de nouvelle génération, divers efforts sont appliqués à l'identification et la caractérisation du microbiote de la glande mammaire bovine (Falentin et al., 2016). En combinant ces nouvelles connaissances avec les études et les tests de formulations probiotiques, il est possible d'envisager de maintenir ou rétablir l'homéostasie et les bienfaits de cet écosystème (Klostermann et al., 2008 ; Espeche et al., 2009 ; Frola et al., 2012 ; Espeche et al., 2012).

Récemment, notre groupe de recherche a isolée des BL du microbiote mammaire. Dans cette procédure, 165 souches ont été isolées, dont dix souches appartenant aux genres *Lactobacillus* et *Lactococcus* ont été caractérisées pour leur potentiel probiotique. Plusieurs critères ont été adoptés, incluant hydrophobie, auto-agrégation, potentiel inhibiteur contre les agents pathogènes causant des mammites et propriétés d'immunomodulation. Cette étude a démontré que certaines des souches étudiées sont des candidats pour le développement de solutions probiotiques montrant de bons résultats, en particulier en ce qui concerne l'activité anti-inflammatoire (Bouchard et al., 2015).

Bien que des solutions probiotiques pour animaux soient assez largement utilisées, les mécanismes sous-jacents des effets bénéfiques ne sont pas bien caractérisés. Plusieurs hypothèses indiquent que les bactéries probiotiques ont le potentiel de réduire ou d'empêcher la croissance des pathogènes en créant un environnement hostile, en bloquant l'accès des pathogènes aux récepteurs des cellules hôtes, en sécrétant des substances antimicrobiennes, épuisant le milieu des éléments nutritionnels nécessaires aux pathogènes et ou en la modulant la réponse du système immunitaire de l'hôte (Bermudez-Brito et al., 2012 ; Goudarzi et al., 2014).

Les composants de la paroi cellulaire des BL probiotiques suscitent un intérêt croissant car ils constituent des candidats potentiellement impliqués dans ces mécanismes probiotiques. La paroi cellulaire a de nombreuses fonctions dans la croissance, le maintien de la forme et de l'intégrité bactérienne. Le peptidoglycane est considéré comme le principal composant de la paroi cellulaire des bactéries à Gram positif. Il se compose d'une alternance de chaînes d'acides N-acétylglucosamine (GlcNAc) et N-acétylmuramique (MurNAc) reliées entre elles par des liaisons glycosidiques β 1-4. Bien que le peptidoglycane présente une structure de base de la paroi bactérienne, il reste toujours à l'état dynamique dû aux processus constant de synthèse, maturation et dégradation (Chapot-Chartier et Kulakauskas, 2014). Dans le

processus de renouvellement du peptidoglycane, l'équilibre entre les processus de synthèse et de dégradation du peptidoglycane est un élément clé. La régulation de ce processus important peut s'effectuer au niveau transcriptionnel ou post-transcriptionnel (Chapot-Chartier, 2010 ; Chapot-Chartier et Kulakauskas, 2014).

Les autolysines sont des enzymes connues pour hydrolyser liens spécifiques du peptidoglycane (Vollmer et al., 2008 ; Chapot-Chartier, 2010). La modification de la paroi cellulaire des bactéries par ces enzymes a été considérée comme un moyen pour les bactéries pathogènes d'échapper au système immunitaire de l'hôte (Davis et Weiser, 2011). Les fragments de peptidoglycane ont en effet sur la capacité de moduler la réponse immunitaire de l'hôte. Ces fragments, appelés motifs moléculaires associés aux microbes (MAMP) sont reconnus par des récepteurs de reconnaissance des motifs moléculaires (PRR) présents dans la cellule hôte, tels que les récepteurs NOD ou TLR (Wells, 2011). L'impact du peptidoglycane de bactéries probiotiques sur la modulation de la réponse immunitaire a rarement été rapportée (Mokrozub et al., 2015). Par contre, ce phénomène a été bien décrit chez les bactéries pathogènes. La reconnaissance par l'hôte de *S. aureus*, lorsqu'il est extracellulaire (i.e. non internalisé), a été déterminée comme résultant de la liaison des fragments de peptidoglycane aux récepteurs de type Toll 2 (TLR2) (Dziarski et Gupta, 2005), alors que la reconnaissance de *S. aureus* intracellulaire implique plus probablement les récepteurs NOD1 et NOD2 (Travassos et al., 2004).

La paroi bactérienne comprend aussi, en surface, certaines protéines qui ont traversé la membrane cytoplasmique et se trouvent liées au peptidoglycane. Cette localisation à la surface exige que de telles protéines possèdent deux domaines. Le premier est le peptide signal, un motif situé dans la région N-terminale de la protéine qui permet la sécrétion généralement par la voie Sec de sécrétion (Tjalsma et al., 2004). Le deuxième est un motif qui dirige l'ancrage des protéines de manière covalente ou non covalente sur la surface de la cellule bactérienne. Les protéines ancrées de façon covalente au peptidoglycane présentent une structure CWA (pour Cell Wall Anchor) dans la région C-terminale, comprenant un motif LPXTG (leucine, proline, un acide aminé quelconque, thréonine et glycine), suivie par une région hydrophobe et une queue d'acides aminés chargés positivement (Cascioferro et al., 2014 ; Cascioferro et al., 2015). Chez les bactéries à Gram-positif, ces protéines LPXTG sont ancrées dans la paroi cellulaire bactérienne par l'action de thiol transpeptidases liées à la membrane, et appelées sortases. Ces enzymes reconnaissent le motif LPXTG, le clivent entre les résidus thréonine-glycine et catalysent une liaison entre le groupe carboxyle du résidu de thréonine C-terminal et un groupe amine du peptidoglycane (Muñoz-Provencio et al., 2012).

Récemment, plusieurs études ont démontré l'influence des composants de la surface cellulaire des BL sur la capacité d'adhésion et auto-agrégation bactérienne (Mokrozub et al., 2015). La surface des cellules BL est recouverte par des acides téïchoiques, des protéines de surface et des polysaccharides (Sengupta et al., 2013). Les protéines de surface ne sont donc pas les seuls facteurs impliqués dans ces propriétés, cependant, elles jouent des rôles importants qui aident à comprendre cette interaction complexe avec l'hôte (Li et al., 2015).

Des études ont été effectuées afin de déterminer le rôle des protéines de surface dans l'interaction avec l'hôte. Chez la souche *Lactobacillus casei* BL23, l'analyse par spectrométrie de masse a révélé la présence de protéines de réponse au stress (GroEL, CLPL), le facteur d'élongation de traduction (EF-Tu, EF-G), et des enzymes impliquées dans la voie de la glycolyse comme l'énolase et la glycéraldéhyde-3-phosphate déshydrogénase (GAPDH) sur sa surface. Postérieurement, des essais *in vitro* avec des protéines énolase et GAPDH ont montré une activité de liaison de ces protéines à la fibronectine et au collagène, ce qui renforce l'idée que *L. casei* BL23 présentent des protéines *moonlighting* sur sa surface (Muñoz-Provencio et al., 2011).

Le potentiel probiotique de *L. casei* BL23 et, notamment, sa capacité à moduler le système immunitaire ont été étudiés dans un environnement intestinal humain (Rochat et al., 2007 ; Lee et al., 2015). Récemment, le potentiel probiotique de *L. casei* BL23 dans le traitement du cancer colorectal induit par le 1,2-diméthylhydrazine a également été démontré dans un modèle murin. La réponse immunitaire des lymphocytes T et la production de cytokines ont été évaluées après incubation des bactéries avec des cellules spléniques provenant de souris non traitées. La protection de *L. casei* BL23 semble liée à la modulation des lymphocytes T régulateurs par une réponse immunitaire de type Th17 accompagnée de l'expression d'IL-6, IL-17, IL-10 et TGF-β dans les splénocytes traités avec des bactéries et de l'expression d'IL-22 *in vivo* (Lenoir et al., 2016).

L. casei est une espèce de BL retrouvée au sein du microbiote mammaire bovin. Son potentiel probiotique dans le contexte mammite a été étudié par Bouchard et coll. en 2013 et sera discuté plus en détail ci-dessous.

3. PROBLEMATIQUE DE LA THÈSE

En 2013, notre équipe a évalué la capacité de trois souches de *L. casei* à inhiber l'adhésion et l'internalisation de *S. aureus* N305 dans les cellules épithéliales mammaires bovines (CEMb). Parmi les souches évaluées dans cette étude, *L. casei* BL23 a été choisie, car

c’est une souche bien caractérisée et dont la séquence génomique est disponible. Dans cette étude, nous avons observé que BL23 a la capacité d'inhiber l'internalisation de *S. aureus* N305. Des expériences préliminaires pour déterminer quels mécanismes pourraient être impliqués dans cet antagonisme ont montré que *L. casei* doit être vivant pour observer cette inhibition. De plus, un contact direct de *L. casei* avec les CEMb et / ou *S. aureus* est nécessaire (Bouchard et al., 2013) .

L’antagonisme de *L. casei* vis-à-vis de l'internalisation de *S. aureus* implique probablement un ou plusieurs mécanismes combinés comprenant la modulation du système immunitaire de l'hôte, la compétition pour les sites d'invasion et la modulation de l'expression de la virulence de *S. aureus*. Plusieurs études ont démontré l'influence des composants de la surface cellulaire dans les propriétés probiotiques. Ainsi, notre hypothèse est que les protéines de surface de *L. casei* BL23 sont impliquées dans ce processus d'antagonisme d’internalisation de *S. aureus*. Les protéines de surface sont particulièrement intéressantes en raison de leur rôle important dans l'interaction des bactéries avec les cellules hôtes. Toutefois, l'évaluation de l'implication de chaque protéine présente à la surface de *L. casei* serait trop longue. Une approche alternative pour cette évaluation est l'étude du rôle des sortases dans cet antagonisme.

Les sortases sont impliquées dans l'ancrage de plusieurs protéines dans la paroi cellulaire bactérienne. En testant l’implication des sortases, nous testons l’implication de leurs substrats, les protéines CWA. Pour cela, l'approche adoptée a été d'utiliser des souches mutantes de *L. casei* BL23 dont un ou plusieurs gènes sortases (*srt*) ont été inactivés. Nous avons testé l’impact de ces mutants sortase dans l'inhibition de l'invasion de *S. aureus* dans des cellules épithéliales mammaires bovines. En parrallèle, les changements dans le profil des protéines ancrées à la surface bactérienne de *L. casei* BL23 sauvage et de ses mutants ont été analysés de manière à identifier les déterminants potentiels de cette inhibition.

Outre leur capacité à inhiber la colonisation des tissus par les pathogènes, les probiotiques ont souvent la capacité à interférer avec la réponse immunitaire innée. Afin d’explorer plus largement le potentiel probiotique de *L. casei* BL23 dans le contexte mammaire, nous avons amorcé l’étude de l’impact de cette souche sur la réponse immunitaire innée des cellules épithéliales bovines.

Ces travaux sont présentés sous forme d’articles dans la section Résultats de ce manuscrit.

CAPÍTULO 1

RESUMO

Os probióticos são considerados como uma estratégia alternativa para o controle de diversas doenças. Nós nos interessamos por essa estratégia para o controle da mastite em bovinos. Para uma melhor compreensão do potencial probiótico no contexto da mastite, nós recentemente exploramos a capacidade de *Lactobacillus casei* BL23 em competir com *Staphylococcus aureus* pela adesão e internalização em células epiteliais mamárias bovinas (CEMb). Nós constatamos que *L. casei* BL23 exerce um efeito antagônico sobre a internalização de *S. aureus* em CEMb. Nós postulamos que a inibição da internalização pode estar baseada na interação entre os componentes da superfície de *L. casei* BL23 e CEMb. Os componentes de superfície foram escolhidos como objeto desse estudo devido à sua importância durante a interação com o hospedeiro. Nesse estudo, nós avaliamos mais especificamente se as sortases e seus substratos podem contribuir para a capacidade de inibir a internalização de *S. aureus* em CEMb. O impacto das mutações sortases sobre a superfície celular de *L. casei* BL23 foi avaliado pela análise do proteoma de superfície, morfologia celular e resistência ao estresse oxidativo. Os resultados obtidos validam nossa hipótese da participação dos componentes de superfície de *L. casei* BL23, abrindo pistas sobre os diferentes componentes da superfície envolvidos nesse potencial inibitório da BL23, pistas que serão validadas

RÉSUMÉ

Les probiotiques sont envisagés comme une stratégie alternative plausible pour le contrôle de diverses maladies. Nous nous intéressons à cette stratégie pour le contrôle de la mammite chez les bovins. Pour une meilleure compréhension du potentiel probiotique en contexte mammite, nous avons récemment exploré la capacité de *Lactobacillus casei* BL23 à rivaliser avec *Staphylococcus aureus* pour l'adhésion et l'internalisation dans les cellules épithéliales mammaires bovines (CEMb). Nous avons constaté que *L. casei* BL23 exerce un effet antagoniste sur l'internalisation de *S. aureus* dans les CEMb. Nous postulons que l'inhibition de l'internalisation peut être basée sur l'interaction entre les composés de surface de *L. casei* BL23 et les CEMb. Les composés de surface ont été choisis comme objet de cette étude en raison de leur importance dans l'interaction avec l'hôte. Dans cette étude, nous avons plus spécifiquement évalué si les sortases et leurs substrats pouvaient contribuer à la capacité d'inhiber l'internalisation de *S. aureus* dans les CEMb. L'impact des mutations sortases sur la surface cellulaire de *L. casei* BL23 a été évalué par l'analyse du protéome de surface, de la morphologie cellulaire et de la résistance au stress oxydatif. Les résultats obtenus valident notre hypothèse d'un rôle des composants de surface de *L. casei* BL23, et ouvrent des pistes sur les différentes composantes de la surface impliquées dans le potentiel inhibiteur de BL23, pistes qui seront à valider

Contribution of Cell Surface Components to *Lactobacillus casei* Inhibition of *Staphylococcus aureus* Internalization into Bovine Mammary Epithelial Cells

Short Title: Determinants of *L. casei* Inhibition of *S. aureus* Internalization into bMEC

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Abstract

Probiotics have been considered as a promising strategy to prevent various diseases in both humans and animals. This approach has gained interest in recent years as a potential means to control bovine mastitis. In a previous study, we found that several *L. casei* strains, including BL23, were able to inhibit the internalization of *S. aureus*, a major etiologic agent of mastitis, into bovine mammary epithelial cells (bMEC). This antagonism required a direct contact between *L. casei* and bMEC or *S. aureus*, suggesting the inhibition relied on interactions between *L. casei* cell surface components and bMEC. Sortases are responsible for the anchoring of proteins bearing an LPXTG motif on the bacterial cell wall, and cell wall-anchored proteins reportedly play an important role in bacteria-host cell interactions. In this study, we investigated the impact of four sortase mutants, *srtA1*, *srtA2*, *srtC1* and *srtC2*, and a double mutant (*srtA1-srtA2*) on the inhibitory potential of *L. casei* BL23 against *S. aureus* invasion, their colonization capacities and their surface properties. All the *srt* mutants tested presented a reduced inhibition capacity, the most pronounced effect being observed with the *srtA2* mutant. A lower internalization capacity of *L. casei* BL23 *srtA2* into bMEC was also observed. This was associated with several changes at the surface of *L. casei* BL23 *srtA2* compared to the wild type strain, including changes in the cell surface exposed proteins. These changes affected the abundance at the cell surface of LPXTG proteins as well as that of moonlighting proteins. Modifications of cell wall thickness in relation to altered peptidoglycan and wall polysaccharide biosynthesis were also observed. These results strongly support the role of cell surface components in *L. casei* inhibition against *S. aureus* internalization. Deciphering the exact contribution of the sortases and their targets in the inhibition will require further investigation.

Introduction

The increasing amount of data on the relationship between host health and microbiota composition has raised a real interest in the development of probiotic solutions for human and animal health, considering that these probiotics could restore a balanced microbiota and, consequently, ecosystem homeostasis. They can also exert or contribute to a barrier effect with regard to pathogen colonization and its associated symptoms [1-3]. In this context, lactic acid bacteria (LAB) are candidates of choice due to their GRAS status (Generally Recognized as Safe) and to the well-documented beneficial effects they exert on intestinal and vaginal human ecosystems [4-6].

Probiotic solutions have been considered as a promising strategy for the control of various diseases in humans [7, 8] and in animals [9] as well. They are of special interest in animal health and are regarded as an alternative means to reduce massive antibiotic use against infectious diseases, including ruminant mastitis in dairy farms [9-13]. *Staphylococcus aureus*, a Gram-positive opportunistic pathogen, is one of the main pathogens involved in mastitis, responsible for great economic losses [14, 15].

To explore the probiotic potential of LAB in a mastitis context, we recently tested the ability of three *Lactobacillus casei* strains, including the well-characterized probiotic strain BL23, to compete with *S. aureus* for bovine mammary epithelial cell (bMEC) adhesion and internalization [16]. In this study, we found that *L. casei* antagonism with regard to *S. aureus* internalization into bMEC required a direct contact between *L. casei* and bMEC or *S. aureus*. We thus postulated that internalization inhibition relied on interactions between *L. casei* cell surface components and bMEC. One of the main mechanisms involved in *S. aureus* internalization relies on the interaction between *S. aureus* fibronectin-binding protein and integrin α5 β1 via fibronectin bridging [17, 18]. *L. casei* produces a fibronectin binding protein (FbpA). We thus postulated that inhibition relied on a competition for fibronectin

attachment through the interaction between *L. casei* FbpA and fibronectin. To test this hypothesis, we evaluated the inhibition capacity of *L. casei* BL23 *fbpA* [19], and we found that it inhibited *S. aureus* internalization at a level similar to that of the wild type (wt) strain, suggesting that FbpA was not involved in inhibition mechanisms (unpublished results). Alternatively, we could not exclude the possibility that other BL23 proteins exhibiting fibronectin-binding activities contributed to this competition.

This study aimed at demonstrating the involvement of cell surface components in the inhibition capacities of *L. casei* with regard to *S. aureus* internalization and at identifying some candidates that might be involved in this phenomenon. Many cell surface components are known to interact with host cells in *L. casei,* so it would therefore be impossible to comprehensively investigate each one of them. We thus evaluated here the inhibition capacities of *L. casei* BL23 sortase mutants [20]. These enzymes are involved in the processing of cell wall-anchored (CWA) proteins. Sortases recognize the LPXTG motif characteristic of CWA proteins, cleave and covalently bind the mature moiety of the protein to the peptidoglycan. Four genes encoding sortases have been identified in *L. casei* BL23, *srtA1, srtA2, srtC1* and *srtC2*. They potentially control the anchoring of 23 proteins that harbor the LPXTG motif in the C-terminal region. Eight of these proteins exhibit adhesion-related functions. Furthermore, the *srtA1-srtA2* double mutant and the *srtA2* mutant show a reduced ability of adhesion to Caco-2 cells [20]. We thus hypothesized that sortases and their substrates could contribute to the inhibition capacities of *L. casei* against bMEC colonization by *S. aureus*. The impact of each single sortase mutant and the *srtA1-srtA2* double mutant on the inhibition of *S. aureus* colonization and on the colonization capacities of *L. casei* BL23 on bMEC was explored. The impact of sortase mutations on the cell surface was monitored through analysis of the cell surface proteome and cell shape, revealing several bacterial surface components that are likely to contribute to the inhibitory potential of *L. casei*.

Material and Methods

Bacterial strains and culture conditions

Lactobacillus casei BL23 [21] wild type and the previously constructed mutants, *srtA1*, *srtA2*, *srtC1*, *srtC2*, *srtA1-srtA2* [20], were used. *S. aureus* Newbould 305 (hereafter referred to as N305), a well-characterized strain of *S. aureus* isolated from bovine mastitis [22], was used for inhibition tests.

For *S. aureus*, subculture was carried out in brain-heart infusion medium (BHI; pH 7.4; BD, Le Pont de Claix, France) at 37°C under agitation (180 rpm), and *L. casei* strains were sub-cultured in Man, Rogosa and Sharpe medium (MRS; pH 6.8; BD) at 37°C without shaking. Subcultures were washed once with phosphate-buffered saline (PBS) and suspended at different concentrations in Dulbecco's modified Eagle's medium (DMEM; pH 7.4; D. Dutscher, Brumath, France) for invasion assays, or in ultra-filtered (UF) milk medium for enzymatic shaving of surface experiments. Alternatively, subcultures for invasion assays were performed on ultra-filtered milk medium so as to be in agreement with conditions used for enzymatic shaving of surface experiments. Whenever necessary (i.e., for *L. casei* BL23 mutant strains), erythromycin (Sigma Aldrich, Saint Louis, USA) was added to the culture at a final concentration of 5 µg/mL.

Bacterial concentrations in subcultures were estimated by spectrophotometric measurements at 600 nm with a VWR V-1200 spectrophotometer. They were further confirmed by determination of the bacterial population using a micromethod, as previously described [23]. The *S. aureus* population (in CFU/mL) was determined on mannitol salt agar (MSA; D. Dutscher) after 24 h of incubation at 37°C. The *L. casei* population was determined on MRS (pH 5.4) and incubated anaerobically for 48 h at 30°C in an anaerobic jar.

Mammary epithelial cell culture

The bovine mammary epithelial cell line (bMEC) MAC-T (Nexia Biotechnologies, Quebec, Canada) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (D. Dutscher) containing 10% fetal calf serum (FCS) (D. Dutscher), 100 U/mL penicillin (Ozyme, *Montigny*-le-*Bretonneux*, France), 10 mg/mL streptomycin (Ozyme) and 5 µg/mL insulin (Sigma Aldrich) in T75 cell culture flasks (Starlab, Orsay, France). Cells were incubated at 37°C in a humidified incubator with 5% carbon dioxide (CO_2). They were cultured to a confluent monolayer, treated with 0.05% trypsin (Sigma Aldrich) and resuspended in fresh DMEM to obtain a final concentration of 2×10^5 cells/mL.

Adhesion and internalization assays were performed in 12-well plates with 2×10^5 cells seeded per well. Cells were incubated in a humidified incubator with 5% CO_2 at 37°C overnight to obtain a confluent monolayer.

Internalization assays

Internalization assays were performed according to Bouchard et al. (2013) [16]. Confluent monolayers of MAC-T cells (at 2.5×10^5 cells/well) were washed twice with phosphate-buffered saline and incubated for 2 hours at 37°C and 5% CO_2 with 1 mL of *L. casei* BL23 wt or mutant strains resuspended in DMEM, with a multiplicity of infection (MOI) of 400:1 or 2,000:1. For internalization inhibition assays, *S. aureus* N305 (MOI 100:1) and *L. casei* strains were co-incubated with cells for two hours. Cells were then washed four times with PBS and incubated with DMEM containing 100 µg/mL gentamicin (Pan Biotech, Aidenbach, Germany) for two additional hours in order to kill adhered extracellular bacteria. The MAC-T cell monolayer was then washed four times with PBS, treated with trypsin, centrifuged for 5 minutes at 800 x g and lysed with triton at 0.01%. Internalized bacterial populations were determined using a micromethod. The *L. casei* population was determined following 48 h

incubation at 30°C on MRS (pH 5.4) in an anaerobic jar. The *S. aureus* population was determined on mannitol salt agar after 24 h of incubation at 37°C.

For internalization inhibition assays, internalization of *S. aureus* was used as a reference. The inhibition rate of internalization was defined as the internalized *S. aureus* population in the presence of *L. casei* with respect to the internalized *S. aureus* population in the reference experiment.

Enzymatic shaving of surface proteins

Enzymatic shaving of *L. casei* was adapted from Le Marechal et al. (2015) [24]. *L. casei* was cultured in ultra-filtered milk medium [25] until the stationary growth phase (48 h). This medium was retained for technical reasons since cultures of *L. casei* on MRS led to contaminating signals in nano-LC-MS analysis in relation to the presence of Tween 20. Moreover, ultra-filtered milk medium contains fewer proteins, which resulted, once again, in fewer "contaminating signals". The bacteria were centrifuged (8,000 x g, 10 min, 4°C), washed three times in one volume of PBS (pH 8.5) containing 5 mM dithiothreitol (DTT) (Sigma Aldrich) and resuspended in 0.5 mL of the same buffer. The 0.5 mL suspension containing 2 x 10^{10} bacteria was treated with 4 mg/mL Sequencing Grade Modified Trypsin (V5111, Promega, Madison, WI, USA) for 1 hour at 37°C with gentle agitation (180 rpm) ("shaving"). Bacteria were then removed by centrifugation (8,000 x g, 10 minutes, 4°C) and the supernatant was filtered (0.2 µm filter, Nalgene, Rochester, USA). The viability of *L. casei* was checked after shaving using a micromethod. For a complete digestion of the peptides released by shaving, the supernatant was further treated with 1 µg of trypsin for 16 hours at 37°C and 120 rpm. Trypsin digestion was stopped by adding trifluoroacetic acid (TFA) to a final concentration of 0.15% (v/v), and the supernatants containing peptides were

then concentrated in a Speed-Vac concentrator prior to nano-LC–MS/MS analysis. Shaving was performed in triplicate on three independent cultures.

Nano LC-MS analyses

Experiments were performed using a nano RSLC Dionex U3000 system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a nano- electrospray ion source. A preliminary sample concentration step was performed on a C18 pepMap100 reverse-phase column (C18 column: inner diameter (i.d.): 300 µm; length: 5 mm; particle size: 5 µm; pore size: 100 Å; Dionex, Amsterdam, the Netherlands). Peptide separation was performed on a reversed-phase column (PepMap RSLC C18: i.d.: 75 µm; length: 150 mm; particle size: 3 µm; pore size: 100 Å; Dionex), with a column temperature of 35°C, using solvent A (2% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water) and solvent B (95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water). Peptides were separated using a gradient of 5 to 35% of solvent B over 70 min, followed by 35 to 85% of solvent B over 5 min at a flow rate of 0.3 µL/min. Eluted peptides were directly electrosprayed into the mass spectrometer operating in positive ion mode with a voltage of 2 kV. Spectra were recorded in full MS mode and selected in a mass range 230-2000 m/z for MS spectra with a resolution of 70,000 at m/z 200. For each scan, the ten most intense ions were selected for fragmentation. MS/MS spectra were recorded with a resolution of 17,500 at m/z 200, and the parent ion was subsequently excluded from MS/MS fragmentation for 20 s. The instrument was externally calibrated according to the supplier's instructions.

Peptides were identified from the MS/MS spectra using X!Tandem pipeline software (Plateforme d'Analyse Protéomique de Paris Sud-Ouest (PAPPSO), INRA, Jouy-en-Josas, France, http://pappso. inra.fr). The search was performed against a database composed of (i) a

portion of the UniProtKB database corresponding to *L. casei* BL23 (http://www.uniprot.org/taxonomy/543734), and (ii) an in-house database composed of major milk and egg proteins taken from www.uniprot.org (207 proteins in total). The latter was used to remove signals associated with milk proteins since cultures were performed on milk-derived medium. Database search parameters were specified as follows: trypsin cleavage was used and the peptide mass tolerance was set to 10 ppm for MS and 0.05 Da for MS/MS. Oxidation of methionine and phosphorylation on threonine, serine and tryptophan were selected as a variable modification. For each peptide identified, a minimum score corresponding to an e-value below 0.05 was considered as a prerequisite for peptide validation.

Transmission electron microscopy

L. casei was grown in ultra-filtered milk medium until the stationary phase of growth so as to be in accordance with growth conditions used for enzymatic shaving of surface experiments. Bacteria were recovered by centrifugation (6,000 x g, 5 min) and fixed for three hours with gentle agitation (150 rpm) in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde. Bacteria were then washed four times and stored at 4°C overnight in a cacodylate buffer. Post-fixation of the bacteria was performed in a cacodylate buffer containing 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) for 1 hour, and bacteria were then embedded in 1.5% agar (Biokar Diagnostics, *Beauvais Cedex, France*). Progressive dehydration was performed with ethanol at concentrations of 50-100% and cells were progressively embedded in Epon (Electron Microscopy Sciences).

Transmission electron microscopy was performed at the MRic platform (University of Rennes 1, Rennes, France). Thin sections of 90 nm were prepared with diamond knives on an

Ultracut, placed on cupper grids, stained with 2% uranyl acetate, and subsequently analyzed with JEOL 1400 TEM (Jeol, Tokyo, Japan) operating at 120 kv accelerating voltage.

Digital images were acquired using the Gatan SC1000 Orius® CCDcamera (4008 x 2672), set up with Gatan Digital Micrograph™ imaging software (Gatan, Pleasanton, CA, USA). TEM images were performed at the Microscopy Rennes Imaging Center platform (MRic-MET, University of Rennes 1, Rennes, France). The same procedure was used to visualize *L. casei* and/or *S. aureus* internalized into bMEC, starting from 2.5 x 10^5 cells.

Image analysis was performed using ImageJ, version 1 [26]. Two measurements of the bacterial wall thickness were made, with a total of ten measurements in each of the three biological replicates.

Assessment of oxidative stress resistance

Resistance of *L. casei* wt and mutant strains to oxidative stress was evaluated through exposure to hydrogen peroxide (H_2O_2). Strains were grown on MRS and were harvested in the exponential phase (OD600 nm: ~ 0.8) or stationary phase (24 h) of growth. H_2O_2 (30% w/v, Sigma Aldrich) was added to 14 mL of culture to a final concentration of 0.1% and 0.2% for the exponential phase and 0.25% and 0.5% for the stationary phase, and incubated at 37°C without agitation. Samples were collected at time 0 (without addition of H_2O_2), and at 10, 20 and 30 minutes of exposure in order to evaluate the surviving *L. casei* population. H_2O_2 was eliminated by adding catalase (Sigma Aldrich) at a final concentration of 10 U/mL and the *L. casei* population was determined by plating serial dilution on MRS and incubating plates for 48 h at 37°C.

Analysis of the auto-aggregation capability

The auto-aggregation test was performed according to Ocaña and Nader-Macias (2002) [27] with some modifications. *L. casei* was cultured in ultra-filtered milk medium for 48 hours at 37°C and the percentage of auto-aggregation was calculated as follows:

$$\text{Auto-aggregation (\%)} = [1 - (OD_{suspenssion}/OD_{total})] \times 100$$

where $OD_{suspension}$ is the optical density (OD) of the cell suspension at 600 nm after 48 hours of *L. casei* growth under static conditions, and OD_{total} is the cell suspension OD measured on the same culture after homogenization. The total bacterial population was also determined by CFU counting to see if differences in OD were related to differences in auto-aggregation or growth capacities in these conditions.

Statistical analysis

Statistical analysis of the bacterial surface proteome was performed as follows: every peptide identified by tandem mass spectrometry after enzymatic shaving of surface proteins was quantified using free MassChroQ software [28] before data treatment and statistical analysis within R software (R 3.2.2, Project for statistical computing). A specific R package called 'MassChroqR' was used to automatically filter dubious peptides for which the standard deviation of retention time was greater than 40 s and to regroup peptide quantification data into proteins.

Three different and complementary methods of analysis were used based on peak counting, spectral counting and XIC (Extracted Ion Chromatogram).

For peak counting and spectral counting analysis, a non-parametric Kruskall-Wallis H test was performed on proteins. A minimum difference of two quantified peaks between strains was retained for peak counting. Proteins with a p-value < 0.05 were considered to be significantly different.

For XIC-based quantification, normalization was performed to take possible global quantitative variations between LC-MS runs into account. For each LC-MS run, the ratio of all peptide values to their value in the chosen reference run was computed. Normalization was performed by dividing peptide values by the median value of peptide ratios. Peptides shared between different proteins were automatically excluded from the dataset, as were peptides present in less than 80% of the samples. Missing data were then imputed from a linear regression based on other peptide intensities for the same protein [29]. Analysis of variance was used to determine proteins with significantly different abundances between mutant strains.

Other statistical analyses were performed with GraphPad Prism software, version 5.01 [30]. Differences between groups were assessed by one-way ANOVA, followed by Bonferroni's Multiple Comparison Test and Student’s Test, considering a P value of less than 0.05. Each experiment was conducted in biological triplicate.

Results

L. casei srtA2 does not inhibit *S. aureus* internalization into bMEC

The inhibitory potential of *L. casei* BL23 wt and *srt* mutant strains against *S. aureus* N305 internalization into bMEC was evaluated using MOIs of 400:1 and 2,000:1 for *L. casei* and 100:1 for *S. aureus*. No significant difference in the *S. aureus* internalization rate was observed in the presence of *L. casei* BL23 wt and mutant strains at an MOI of 400:1 (data not shown). A significant reduction of 58% of the internalization rate of *S. aureus* into bMEC was observed in the presence of *L. casei* BL23 wt at an MOI of 2,000:1, whereas none of the sortase mutants showed a significant inhibition of *S. aureus* internalization (Fig. 1). A significant and maximum release of inhibition was obtained with the *srtA2* mutant strain when compared to the inhibition observed with the control *L. casei* BL23 wt. In order to be in accordance with enzymatic shaving experiments that were done using UF-medium grown cultures for technical reasons, we further confirmed that similar results were obtained when *L. casei* subcultures were performed on UF-medium instead of MRS prior to invasion assays (data not shown). Results were further confirmed by transmission electron microscopy (TEM) observation (Fig. 2). A greater number of internalized *S. aureus* was observed when cells were infected by *S. aureus* alone (Fig. 2, panels A and B) and in the presence of *L. casei srtA2* (panels E and F), compared to internalized *S. aureus* in the presence of the *L. casei* wt strain (panels C and D).

Reduced internalization of *L. casei srtA2* in bMEC

Adhesion and internalization capacities of *L. casei* BL23 wt and mutant strains were determined on bMEC with an MOI of 400:1 and 2,000:1. No significant difference in the adhesion rate was observed in sortase mutant strains compared to the BL23 wt strain (data not

shown). However, a significant reduction of internalization rates was obtained for *srtA2* (37%) and *srtA2*-*srtA1* (24%) strains compared to BL23 wt with an MOI 2,000: 1 (Fig. 3). Of note, TEM observation of bMEC infected with BL23 wt or srtA2 showed degradation vesicles in greater abundance in the bMEC infected with the *srtA2* mutant strain (Fig. 4). The observed reduction in the *srtA2* mutant internalization may thus result either from a lower capability of internalization into bMEC and or from a lower survival rate of this strain once internalized into the bMEC.

L. casei srtA2 is less resistant to oxidative stress

Oxidative stress is a major stress encountered by bacteria once it has been internalized into eukaryotic cells. Resistance to oxidative stress was evaluated by measuring the resistance of *L. casei* BL23 wt and *srtA2* strains to H_2O_2 exposure in the exponential and stationary phases of growth. No significant difference in survival to H_2O_2 exposure was observed between strains when tested in the exponential phase (data not shown). In contrast, when tested in the stationary phase, *L. casei* BL23 *srtA2* was more sensitive to H_2O_2 exposure than BL23 wt (Fig. 5). The difference was more pronounced after 20 minutes of exposure to 0.5% H_2O_2, as illustrated by a survival rate of approximately 1 x 10^5 CFU/mL of *L. casei* BL23 wt, whereas there was no residual population of the *srtA2* strain.

Altered profile of surface proteins in *L. casei srtA2*

The impact of *srtA2* disruption on the cell surface proteome of *L. casei* BL23 was determined by enzymatic shaving, using three different and complementary methods of analysis, based on peak counting, spectral counting and XIC (Extracted Ion Chromatogram). While the two former methods are based on the detection of spectra and thus reveal proteins that exhibit strong differences between conditions, the latter reveals proteins that are present in both

conditions but with differential abundance. Complete datasets are presented in S1, S2 and S3 Tables. Proteomic data analysis revealed several changes in the cell surface proteome of BL23 *srtA2*. Hence, 57, 144 and 196 proteins were differentially abundant at the cell surface of *L. casei* BL23 *srtA2* compared to the BL23 wt strain using the peak counting, spectral counting and XIC methods, respectively. A selection of proteins differentially abundant at the cell surface between wt and *srtA2* mutant strains and discussed in this work is presented in Table 1. They include the proteins containing the LPXTG motif, those presumably located at the cell surface or involved in cell surface component biosynthesis, or those known to be involved in the interaction with the host, including moonlighting proteins and proteins involved in cellular stress response.

Table 1. Cell surface proteome of *L. casei* BL23 wt and srtA2 mutant as determined by enzymatic shaving.

Protein ID	Gene ID	Protein name	Gene name	Localization	Moonlighting[a]	Ratio *srtA2*/wt[b]			Function
						Peak counting	Spectral counting	XIC	
LPXTG motif containing proteins									
B3W8P4	LCABL_02860	Beta-N-acetylglucosaminidase	*bnaG*	PSE	-			0.50	Physiological processes
B3WA51	LCABL_25040	Internalin-J	*inlJ*	PSE	-			2.35	Unknown
Cell wall/membrane/envelope biogenesis									
B3WF06	LCABL_18780	Bifunctional dimerization/transpeptidase penicillin-binding protein 2B	*pbp2B2*	Secreted	-		3.75	6.27	Peptidoglycan synthesis
B3W8K4	LCABL_02120	UDP-N-acetylmuramyl-tripeptide synthetase	*murE*	Cytoplasmic	-			1.47	Peptidoglycan synthesis
B3WCW2	LCABL_11280	N-acetylmuramoyl-L-alanine amidase	*lys*	Cytoplasmic	Yes		0.00[c]		Cell wall degradation
B3W9B1	LCABL_22130	Tyrosine-protein phosphatase	*wzb*	Cytoplasmic			0.32		Exopolysaccharide biosynthesis
B3W9B2	LCABL_22140	Cell envelope-related transcriptional attenuator	*wzr*	Secreted			-[d]		Exopolysaccharide biosynthesis
B3W978	LCABL_22210	dTDP-glucose 4,6-dehydratase	*rmlB*	Cytoplasmic				3.83	Exopolysaccharide biosynthesis
B3W979	LCABL_22220	dTDP-4-dehydrorhamnose 3,5-	*rmlC*	Cytoplasmic				0.70	Exopolysaccharide

		epimerase							biosynthesis
B3W9D2	LCABL_22340	exopolysaccharide biosynthesis protein	*wze*	Cytoplasmic				0.68	Exopolysaccharide biosynthesis
Cell division									
B3WDV7	LCABL_14770	Septation ring formation regulator ezrA	*ezrA*	PSE	-			2.60	Cell division
B3WEU8	LCABL_18190	Signal recognition particle-docking protein	*ftsY*	Cytoplasmic	-	1.23			Cell division
B3WDY9	LCABL_15090	Cell-division initiation protein (Septum placement)	*divIVA*	Cytoplasmic	-			0.39	Cell division
B3WCN1	LCABL_10450	Cell division protein FtsX	*ftsX*	Cytoplasmic	-		0.21		Cell division
Stress response									
B3WCP7	LCABL_10620	Thioredoxin reductase	*trxB2*	Cytoplasmic	-	1.13		2.65	Oxidative stress response
B3WC30	LCABL_08080	Thiol peroxidase (Hydroperoxide reductase, Peroxiredoxin)	*tpx*	Cytoplasmic	-			1.36	Oxidative stress response
B3WCJ1	LCABL_10060	Glutathione peroxidase	*bsaA*	Cytoplasmic	-			0.47	Oxidative stress response
B3WE57	LCABL_15770	Chaperone ClpB	*clpB*	Cytoplasmic	-		1.72		Heat shock response
B3WAM9	LCABL_26830	ATP-dependent Clp protease, ATP-binding subunit ClpC	*clpC*	Cytoplasmic	-	1.25	1.89		Heat shock response
B3WCR2	LCABL_10770	ATP-dependent Clp protease proteolytic subunit	*clpP*	Cytoplasmic	-	1.58	2.22		Heat shock response
B3WEQ8	LCABL_17790	Protein GrpE	*grpE*	Cytoplasmic	-			0.70	Heat shock response
B3W9A4	LCABL_22060	1,4-alpha-glucan branching enzyme (glycogen branching enzyme)	*glgB*	Cytoplasmic	-			0.64	Carbohydrate metabolism

B3W9A0	LCABL_22020	Glycogen phosphorylase	*glgP*	Cytoplasmic	-	0.71	0.25		Carbohydrate metabolism
Moonlighting proteins								
B3WCW4	LCABL_11300	Glyceraldehyde 3-phosphate dehydrogenase	*gap-1*	Cytoplasmic	Yes			0.44	Carbohydrate metabolism
B3WE98	LCABL_16180	Glyceraldehyde 3-phosphate dehydrogenase	*gapB*	Cytoplasmic	Yes	0.63			Carbohydrate metabolism
B3W7V2	LCABL_05010	Fructose-bisphosphate aldolase	*fba*	Cytoplasmic				0.64	Carbohydrate metabolism

[a] Proteins that are described in the literature as moonlighting proteins.
[b] Ratio of protein abundance between mutant *srtA2* and *L. casei* wt, as determined by three different methods (see Material and Methods).
[c] Detected in wt strain but not detected in *srtA2* mutant.
[d] Detected in the *srtA2* mutant but not detected in the wt strain.

Two proteins containing the LPXTG motif were identified. Internalin J (InlJ), a protein of unknown function, was found to be more abundant in the BL23 *srtA2* surfaceome, whereas Beta-N-acetylglucosaminidase (BnaG), a protein involved in physiological processes, was more abundant in BL23 wt.

Several proteins other than CWA proteins exhibited differential abundance in the cell surface proteome in BL23 *srtA2* and are involved in cell wall synthesis or degradation, exopolysaccharide biosynthesis, as well as in cell division processes. A greater abundance was observed in BL23 *srtA2* for the bifunctional dimerization/transpeptidase penicillin-binding protein 2B (Pbp2B2), UDP-N-acetylmuramyl-tripeptide synthetase (MurE), two proteins involved in exopolysaccharide biosynthesis (RmlB and Wzr), the septation ring formation regulator EzrA, and the signal recognition particle-docking protein (FtsY). On the contrary, a greater abundance was observed in the wt strain for N-acetylmuramoyl-L-alanine amidase, the cell-division proteins DivIVA and FtsX, and four proteins involved in exopolysaccharide biosynthesis (Wzb, RmlC, Wze and Wzd).

In addition, three proteins known as moonlighting proteins were found to be less abundant at the cell surface of BL23 *srtA2*. They included the fructose bisphosphate aldolase and two glyceraldehyde 3-phosphate dehydrogenases. Of note, the N-acetylmuramoyl-L-alanine amidase mentioned above is also known as a moonlighting protein [31].

Finally, another set of differentially abundant proteins included proteins involved in the interaction with the host cell and in the fitness of bacteria within their host. This group is comprised of proteins involved in stress response. Both thioredoxin reductase and thiol peroxidase, involved in oxidative stress response, were more abundant in BL23 *srtA2,* as were the ClpB chaperone, ClpC and ClpP protease. On the contrary, the glutathione peroxidase and the GrpE chaperone were more abundant in BL23 wt. In addition, three proteins involved in

glycogen biosynthesis, GlgB, GlgC and GlgP, were also more abundant in the surfaceome of BL23 wt.

L. casei srtA2 has a thinner cell wall

The impact of *srtA2* disruption on *L. casei* cell morphology was investigated using TEM on BL23 grown on ultra-filtered milk medium until the stationary phase (48 hours). Although no alteration of the cell morphology (e.g., rod shape and length) was observed, a significant reduction (Student's Test; pval = 2.99589E-15) of the cell wall thickness was measured in BL23 *srtA2* compared to the BL23 wt control (Fig. 6). Cell wall thickness was found to be 26.5 +/- 4.6 nm and 16.0 +/- 2.6 nm in *L. casei* BL23 wt and *srtA2* strains, respectively.

L. casei srtA2 presents a greater auto-aggregation ability

The auto-aggregation capacity of *L. casei* BL23 wt and *srtA2* strains was assessed after a growth of 48 h in ultra-filtered milk medium (stationary phase). The auto-aggregation rate of BL23 *srtA2* was slightly but significantly higher compared to that of the wt control, as illustrated by auto-aggregation rates of 50% and 66.67% in *L. casei* BL23 wt and *srtA2* strains, respectively (Fig. 7). The total bacterial population counts were similar in both strains.

Discussion

In this work, we investigated the involvement of cell surface components in the capacity of *L. casei* BL23 to inhibit *S. aureus* internalization into bMEC. We focused on the *L. casei* sortase genes. Sortases specifically ensure the anchoring of CWA proteins in the cell wall of Gram-positive bacteria. The disruption of sortase genes thus directly affects the bacterial surfaceome in terms of CWA protein display and also indirectly affects the surfaceome through other changes in surface components [32]. The effect of sortase gene disruption in *L. casei* BL23 was thus evaluated with regard to surface properties, colonization capacities and inhibition potential against *S. aureus* bMEC invasion. All sortase mutant strains resulted in a reduced *L. casei* BL23 ability to inhibit *S. aureus* internalization into bMEC. The most pronounced effect was observed with the BL23 *srtA2* mutant. The internalization capacity of *L. casei* BL23 *srtA2* alone into bMEC was also reduced. We thus focused on this BL23 *srtA2* mutant to investigate changes in surface properties that may contribute to the loss of inhibition, by combining surface proteome analysis and TEM observations of bacterial cell shape and *L. casei*-infected bMEC with phenotypic characterizations. The *srtA2* mutation resulted in several changes at the bacterial surface, which, for some of them or all together, contribute to the loss of inhibition properties. These changes affect cell surface-exposed proteins beyond the direct substrates of sortases (i.e., proteins containing an LPXTG motif and a C-terminal cell wall anchor structure), moonlighting protein abundance, and they include modifications in the cell wall thickness and biosynthesis as well as in the oxidative stress response.

Some surface proteins are important molecules for colonization and persistence in the ecosystem, as well as for cross-talk between the host cells and the immune system in both pathogenic and probiotic bacteria [33]. Among these surface proteins, some CWA proteins have been described as important proteins since they promote the adhesion or invasion process in eukaryotic cells [34]. In this study, only two proteins bearing the LPXTG motif

were found to be differentially abundant in the BL23 surfaceome. Internalin J (InlJ) was more abundant in *L. casei srtA2*, whereas Beta-N-acetylglucosaminidase (BnaG) was more abundant in *L. casei* BL23 wt. In *Listeria monocytogenes,* InlJ reportedly contributes to virulence but its precise function is not known and it is not directly involved in cell invasion, contrarily to Internalin A and B [35, 36]. Internalin-like proteins have been described in other bacteria, including food-grade bacteria like *L. casei* or *Propionibacterium freudenreichii* [24], but their biological function remains unclear [36, 37]. Of note, *L. casei* InlJ does not contain an N-terminal leucine-rich repeat (LRR) domain, which is characteristic of internalin family members [36]. Whether InlJ in *L. casei* BL23 contributes to cell invasion remains to be determined. BnaG, the second LPXTG motif-containing protein identified here, harbors a GH20_DspB_LnbB-like domain (glycosyl hydrolase family 20 (GH20) catalytic domain of dispersin B (DspB), lacto-N-biosidase (LnbB) and related proteins) [38, 39]. *L. casei* BL23 BnaG was recently characterized. It is an extracellular enzyme involved in the metabolism of lacto-N-triose [40], a compound found in human milk oligosaccharides as well as in the glycan moieties of glycoproteins. The GH20_DspB_LnbB-like catalytic domain is also found in dispersin B, a glycoside hydrolase that hydrolyzes the beta-1,6-linkages of PNAG (poly-beta-(1,6)-N-acetylglucosamine), a major component of the extracellular polysaccharide matrix. This polysaccharide is notably produced by several staphylococcal species, including *Staphylococcus aureus* and *Staphylococcus epidermidis* [41]. It is considered as an important adhesin that facilitates adhesion to biomaterials [38, 42]. Whether BnaG contributes to the inhibition capacity of *L. casei* BL23 with regard to *S. aureus* colonization of bMEC by interfering with intercellular adhesion of *S. aureus* remains to be determined.

Moonlighting proteins are a special class of multifunctional proteins, some of which have functions related to adhesion [43]. Here, three proteins, mainly known for their intracellular role in glycolysis and gluconeogenesis and that exhibit moonlighting functions in other

bacteria [44], were identified with greater abundance in the BL23 wt strain. They include two glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a fructose-bisphosphate aldolase (FBA). GAPDH was found extracellularly and shown to bind to human colonic mucin in *Lactobacillus plantarum* LA 318. Likewise, in the group A *Streptococcus,* GAPDH displayed multiple binding activities to fibronectin, lysozyme, myosin and actin [45, 46]. Likewise, FBA has been found to be surface-exposed in several bacteria, including pathogens such as *Neisseria meningitidis*, which causes meningitis and septicemia [47]. In this species, FBA was shown to contribute to the adhesion to human brain microvascular endothelial and HEp-2 cells.

An additional moonlighting protein, N-acetylmuramoyl-L-alanine amidase, was found in greater abundance in *L. casei* BL23 wt compared to BL23 *srtA2*. N-acetylmuramoyl-L-alanine amidase is an enzyme that cleaves the amide bond between N-acetylmuramoyl residues and L-amino acid residues in bacterial cell walls. This hydrolase was also identified in *Mycobacterium tuberculosis* as an adhesin capable of binding to fibronectin and laminin [48]. The lower relative abundance of all these moonlighting proteins in BL23 *srtA2* may contribute to its altered colonization properties and inhibition capacity.

In addition to surface proteins, other components of the bacterial surface, including peptidoglycan and exopolysaccharides, are known to interact with the host. Peptidoglycan plays an important role in protecting the bacterial structural integrity and allows the covalent or non-covalent anchoring of various structures such as teichoic acids, polysaccharides and proteins [49]. Peptidoglycan fragments, when released after degradation, can induce an immune response in the host cells [50]. Exopolysaccharides (EPS), including capsular polysaccharide, wall PS or secreted ones, have also been shown to be involved in interactions with the host and, notably, interactions with the immune system in both probiotic and pathogenic strains [49, 51]. Electron microscopy analysis showed that the cell wall was

thinner in *L. casei* BL23 *srtA2*. When considering the other changes identified here in the BL23 *srtA2* surfaceome, this suggests a possible imbalance in the bacterial cell wall turnover and/or exopolysaccharide biosynthesis. Three proteins involved in the cell wall synthesis process were differentially abundant on the cell surface in *L. casei* BL23 *srtA2* compared to the wt strain. These include the bifunctional dimerization/transpeptidase penicillin-binding protein 2B (Pbp2B2) and a UDP-N-acetylmuramyl-tripeptide synthetase (MurE) that were more abundant on the BL23 *srtA2* surface. On the contrary, an N-acetylmuramoyl-L-alanine amidase was more abundant in the wt surfaceome. This peptidoglycan hydrolase cleaves specific bonds in peptidoglycan, thus contributing to the insertion of newly synthesized peptidoglycan subunits and to the separation of daughter cells following division [33]. We did not microscopically observe changes in cell size or cell separation, but it can be noted that four proteins involved in cell separation were also differentially abundant on the cell surface on wt and *srtA2* mutant strains. In addition, several proteins encoded within the EPS biosynthesis gene cluster (LCABL_22130 to LCABL_22350) displayed modified abundance in the *srtA2* mutant compared to *L. casei* BL23 wt. They include two proteins involved in NDP-sugar (EPS-precursors) biosynthesis (RmlB, RmlC) and three proteins involved in polysaccharide-chain-length determination (Wzd, Wze, Wzb), which were less abundant in *L. casei* BL23 *srtA2* than in the wt strain, with the exception of RmlB [51, 52]. Wzr was only detected in the *L. casei* BL23 *srtA2* surfaceome. The *wzr* gene is organized in the opposite transcriptional sense and probably encodes a transcriptional regulator whose exact function is unknown. EPSs have been shown to influence intercellular interactions and adhesion to biotic and abiotic surfaces by contributing to the cell surface physicochemical properties and/or by hiding some of the surface-exposed proteins, including some adhesins [53]. EPSs thus play a role in the formation of microcolonies and biofilms. Inactivation of *wzb*, which is involved in polysaccharide-chain-length determination in *L. rhamnosus* GG, resulted in altered biofilm

formation capacities [54]. Whether modulations of peptidoglycan and or EPS biosynthesis account for the thinner cell wall of *L. casei* BL23 *srtA2* remains to be determined. Similarly, the impact of these modifications on *L. casei* BL23 interactions with bMEC is subject to further investigation.

Interestingly, when observing internalized *L. casei* BL23 wt and *srtA2* strains by TEM, a higher number of degradation vesicles were observed in bMEC infected with the mutant strain, suggesting that the lower internalization rate may be due to a lower capacity to invade cells, combined with a lower capacity to survive intracellularly. This was supported by a lower resistance of BL23 *srtA2* to oxidative stress, one of the harmful mechanisms used by eukaryotic cells to reduce bacterial cell viability [55]. In agreement with an impact of the *srtA2* mutation on oxidative stress resistance, three oxidative stress-related proteins, thioredoxin reductase, thiol peroxidase and glutathione peroxidase, were found to be differentially abundant in BL23 wt and *srtA2* surfaceomes. Several additional proteins related to stress response, including chaperones and proteases, were also differentially abundant in BL23 wt and *srtA2* surfaceomes. ClpC was notably shown to contribute to the persistence capacity of *Lactobacillus plantarum* WCFS1 in murine GIT [56]. Finally, three proteins involved in glycogen metabolism were more abundant in the surfaceome of the wt strain. Glycogen metabolism is associated with energy storage and various physiological functions, including colonization and persistence [57]. Alteration of stress response capacity and, consequently, of intracellular survival of *L. casei* BL23 *srtA2* may also result in modifications of its inhibitory capacities against bMEC invasion by *S. aureus*.

In conclusion, *srtA2* mutation resulted in a complete loss of *L. casei* BL23 inhibition capacity with regard to *S. aureus* internalization, supporting the hypothesis of a major role of surface components in this inhibition. The analysis of *srtA2* disruption on BL23 showed that it resulted in pleotropic effects, including several changes in the surface proteome, beyond the

LPXTG substrates, and changes at the cell wall level. Altogether, these results provide numerous presumptions about the functions (cell wall biosynthesis, oxidative stress and adhesion) putatively involved in this inhibition. They therefore open avenues for continuing research into this phenomenon since the candidates identified here could be targets for further characterization of this promising *S. aureus*-*L. casei*-bMEC tripartite interaction.

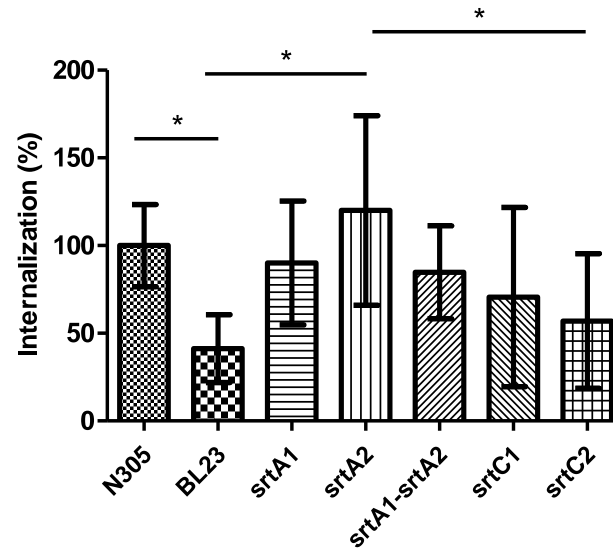

Figure 1.Inhibition of *S. aureus* internalization into bMEC by *L. casei*. Internalization rates of *S. aureus* N305 after 2 h of interaction with bMEC and co-incubation with wt and mutant strains of *L. casei* BL23 at an MOI of 2,000:1. *S. aureus* was used at an MOI of 100:1. The internalization assay of *S. aureus* alone was used as a reference. Internalization rates were then defined as the internalized *S. aureus* population in the presence of the different *L. casei* strains relative to the internalized *S. aureus* population of the reference experiment. Data are presented as means ± standard deviations. Each experiment was done in triplicate, and differences between groups were compared using one-way ANOVA with Bonferroni's Multiple Comparison Test. *: $P < 0.05$.

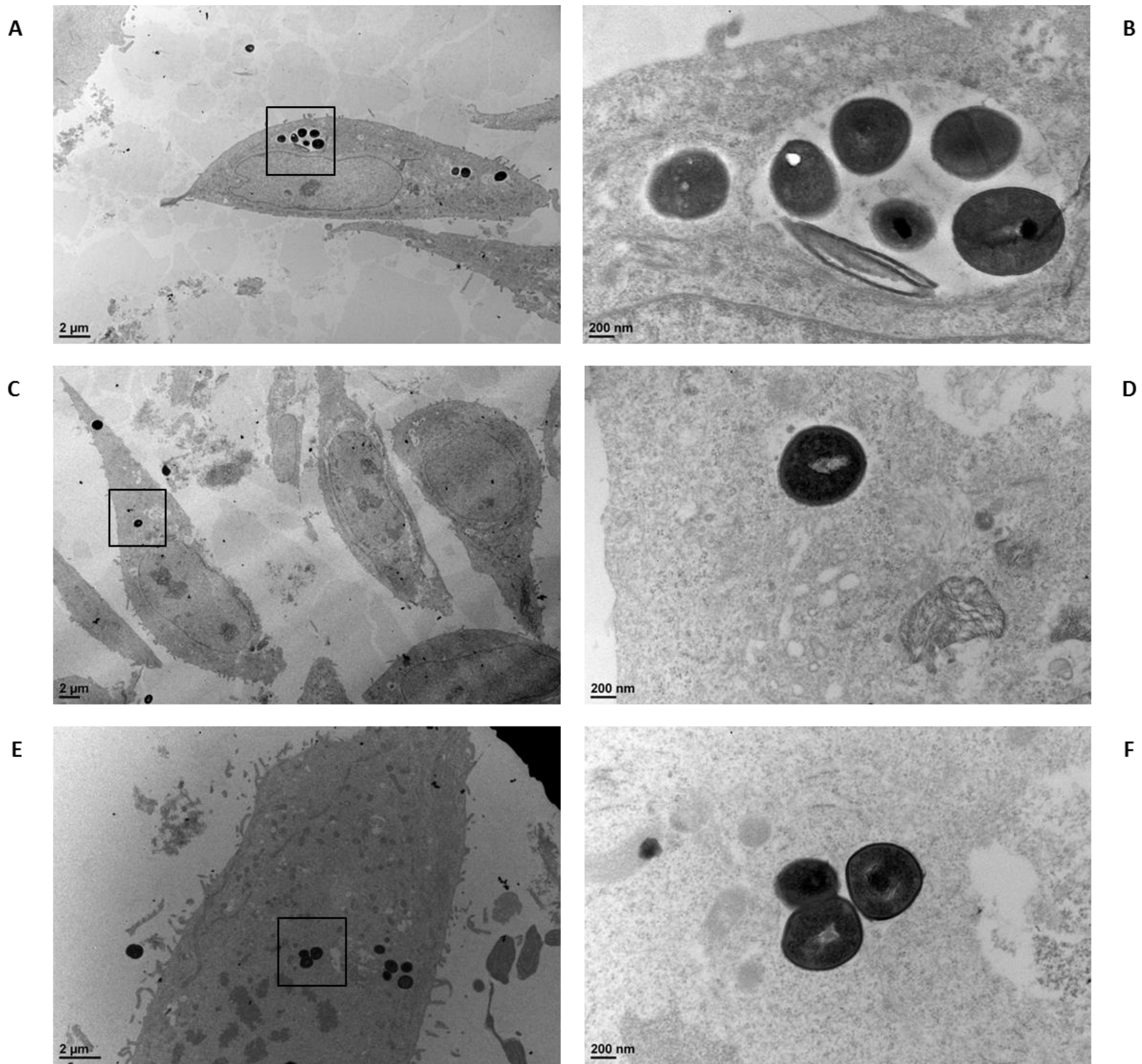

Figure 2. Microscopic observation of internalized *S. aureus*. Internalization of *S. aureus* N305 as observed by transmission electron microscopy. *S. aureus* N305 (at an MOI of 100:1) was incubated for 2 h with bMEC either alone (A, B) or in the presence of *L. casei* BL23 wt (C, D) or *srtA2* mutant (E, F) strains, at an MOI of 2,000:1.

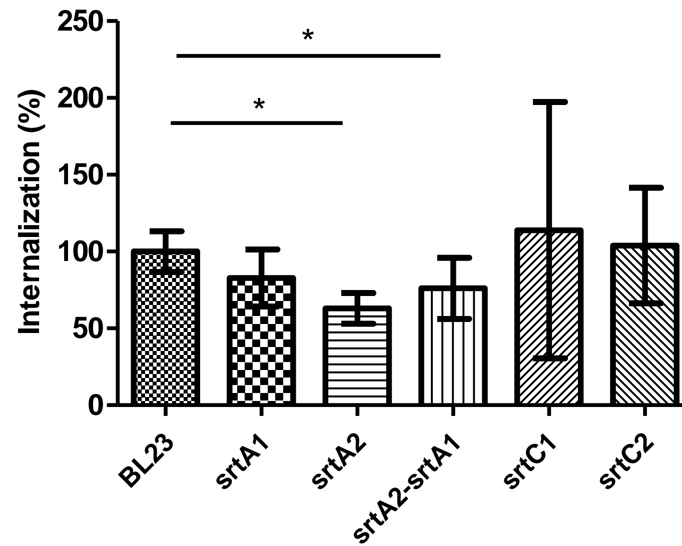

Figure 3. Internalization of wild type and mutant strains of *L. casei* BL23 into bMEC. *L. casei* populations internalized into bMEC were determined after 2 h of interaction at an MOI 2,000:1. The internalization assay of the *L. casei* BL23 wild type (wt) strain was used as a reference. Internalization rates were defined as the internalized population of mutant strains relative to the internalized *L. casei* BL23 wt strain population. Data are presented as mean ± standard deviations. Each experiment was done in triplicate and differences between groups were compared using Student's Test. *: $P < 0.05$.

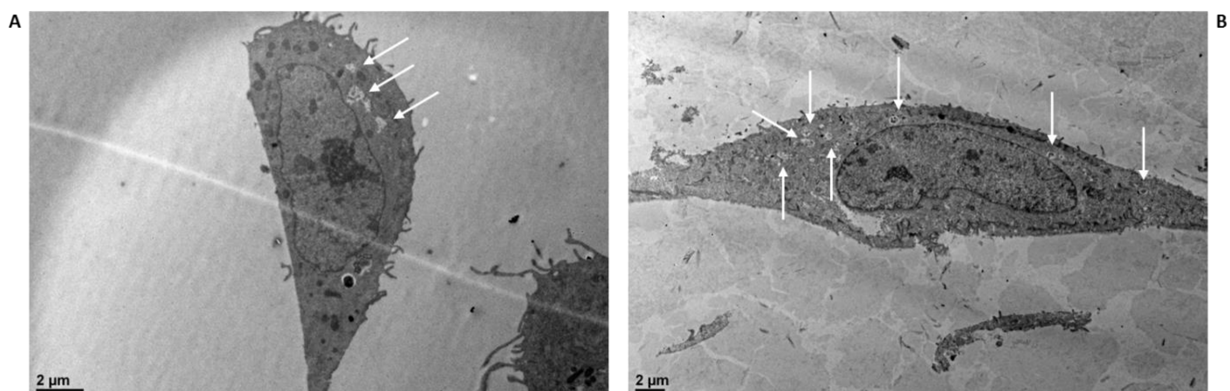

Figure 4. Internalization of *L. casei* BL23 wt (A) and srtA2 mutant (B) strains as observed by transmission electron microscopy. Degradation vesicles (white arrows) were observed in a greater proportion in mutant *srtA2*.

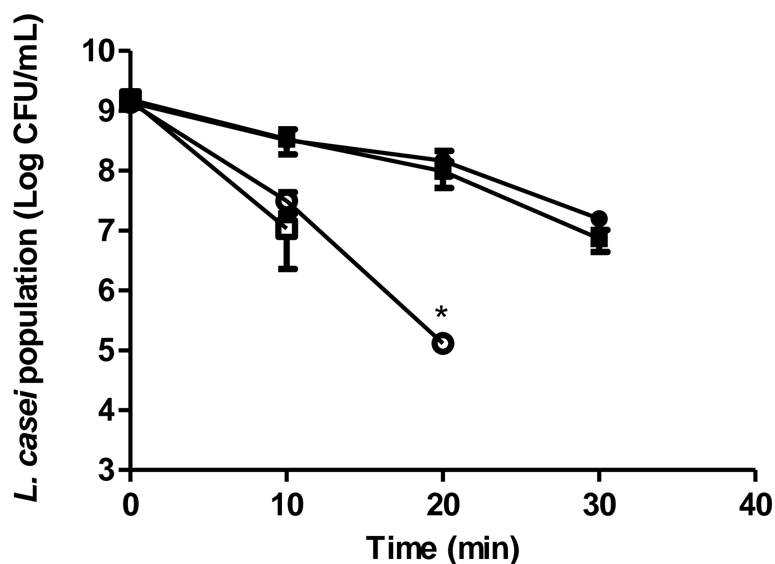

Figure 5. Resistance of *L. casei* BL23 wt and srtA2 strains to H2O2. Resistance of *L. casei* BL23 wt (●, ○) and *srtA2* (■,□) strains to H_2O_2 was evaluated in the stationary phase of growth of *L. casei* (24h - MRS). The residual population was evaluated at 0, 10, 20 and 30 min after exposure to 0.25% (●, ■) and 0.5% H_2O_2 (○,□). Data are presented as means ± standard deviations. Each experiment was done in triplicate, and differences between groups were compared using Student's Test. *: $P < 0.05$.

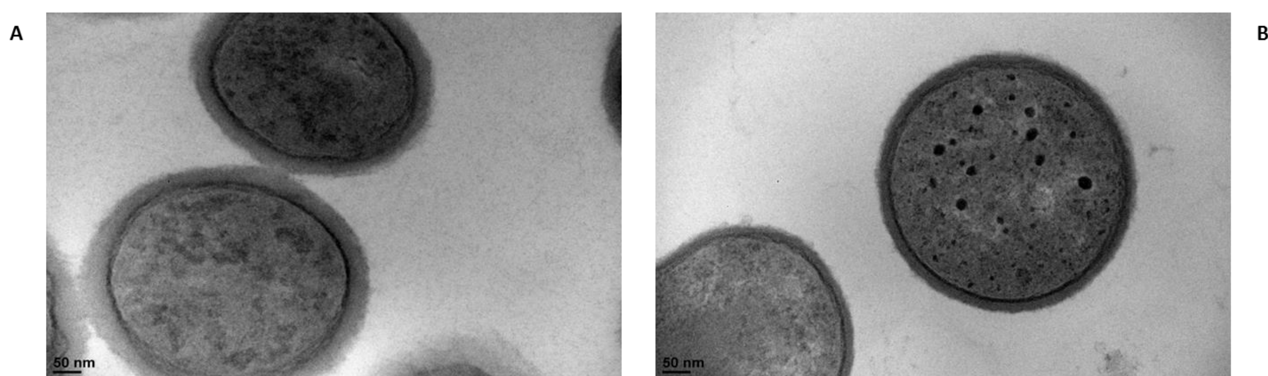

Figure 6. *L. casei* BL23 srtA2 displayed a lower cell wall thickness compared to the wt strain. *L. casei* wt (A) and *srtA2* mutant (B) strains were grown in ultra-filtered milk medium for 48 h and observed by transmission electron microscopy.

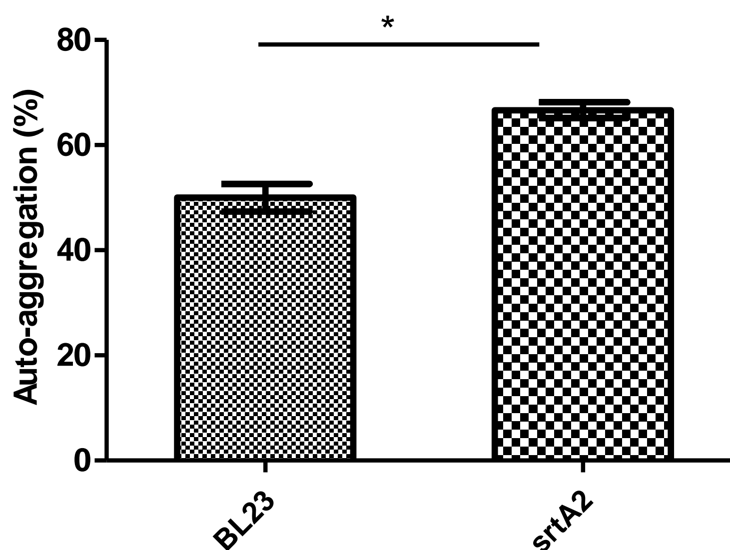

Figure 7. Auto-aggregation capacities of *L. casei* BL23 wt and srtA2 strains. Strains were grown in ultra-filtered milk medium for 48 h at 37°C. Auto-aggregation was evaluated by spectrophotometry (600 nm) and expressed as the auto-aggregation percentage. Cell suspension OD after growth (48 h) and homogenization was used as a reference (100%). Data are presented as means ± standard deviations. Each experiment was done in triplicate, and differences between groups were compared using Student's Test. *: $P < 0.05$.

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CAPÍTULO 2

RESUMO

Embora as soluções probióticas sejam adotadas em diversos contextos, os mecanismos subjacentes aos efeitos benéficos ainda não são completamente caracterizados. Vários mecanismos de ação podem ser mencionados como meios pelos quais os probióticos exercem os seus efeitos, entre os quais, a modulação da resposta imune inata ou adquirida do hospedeiro figura como uma das mais importantes. Essas respostas imunes são mais frequentemente avaliadas através da dosagem de citocinas e defensinas produzidas pelas células do hospedeiro. A imunomodulação em contexto mamário ainda é pouco documentada. Nós recentemente identificamos que *Lactobacillus casei* BL23 foi capaz de inibir a internalização de *Staphylococcus aureus* em células epiteliais mamárias bovinas (CEMb). Além disso, essa linhagem reduziu a secreção de IL-8 em CEMb estimuladas com *Escherichia coli*. Diante disso, nosso objetivo foi determinar se *L. casei* BL23 tem o potencial de modular a resposta imune do hospedeiro no contexto da mastite causada por *S. aureus*. Para isso, diferentes condições de incubação entre *L. casei* BL23 e *S. aureus* com as CEMb foram estabelecidas, a fim de observar o seu potencial imunomodulador através da análise da expressão de diversas citocinas e defensinas por PCR quantitativa. Nós observamos que *L. casei* BL23 exibiu propriedades anti-inflamatórias no contexto da mastite causada por *S. aureus*. Esses resultados caracterizam um trabalho preliminar que ainda necessitará ser complementado antes de sua valorização. As diferentes perspectivas serão apresentadas na discussão geral da tese.

RÉSUMÉ

Bien que des solutions probiotiques soient déjà adoptées dans divers contextes, les mécanismes sous-jacents des effets bénéfiques ne sont pas encore pleinement caractérisés. Plusieurs mécanismes d'action peuvent être évoqués, parmi lesquels la modulation de la réponse immunitaire innée ou acquise de l'hôte représente l'un des plus importants. Ces réponses immunitaires sont le plus souvent évaluées par dosage des cytokines et des défensines produites par les cellules hôtes. L'immunomodulation dans le contexte mammaire est encore peu documentée. Nous avons récemment identifié que *Lactobacillus casei* BL23 était capable d'inhiber l'internalisation de *Staphylococcus aureus* dans les cellules épithéliales mammaires bovines (CEMb). En outre, cette souche réduit la sécrétion d'IL-8 dans les CEMb stimulées par *Escherichia coli*. Par conséquent, notre objectif a été de déterminer si *L. casei* BL23 a le potentiel de moduler la réponse immunitaire de l'hôte provoquée par *S. aureus* en contexte mammite. Pour cela, différentes conditions d'incubation entre *L. casei* BL23 et *S. aureus* avec les CEMb ont été établies afin d'observer leur potentiel immunomodulateur par dosage de l'expression de diverses cytokines et défensines par PCR quantitative. Nous avons ainsi observé que *L. casei* BL23 présente des propriétés anti-inflammatoires dans le contexte de la mammite provoquée par *S. aureus*. Il s'agit ici d'un travail préliminaire qui demandera à être complété avant valorisation. Les différentes perspectives sont evoquées dans la discussion générale de la thèse.

***Lactobacillus casei* BL23 modulates the innate immune response in *Staphylococcus aureus*-stimulated bovine mammary epithelial cells**

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Abstract

Probiotics have been adopted to treat and prevent various diseases. They were shown to be a promising alternative to combat mastitis in dairy cattle. This inflammation of the mammary gland is generally of infectious origin and generates great economic losses in the world. In a previous study, we found that *Lactobacillus casei* BL23 was able to inhibit the internalization of *Staphylococcus aureus,* one of the major pathogens involved in mastitis, into bovine mammary epithelial cells (bMEC). In this study, we further explored the capacity of this strain to interfere with the innate immune response of bovine mammary epithelial cells during *S. aureus* infection. *L. casei* BL23 was able to decrease the expression of several pro-inflammatory cytokines, including IL-6, IL-8, IL-1α, IL-1β and TNF-α, in *S. aureus*-stimulated bMEC. On the other hand, *L. casei* did not impair the induction of defensins such as LAP and DEFβ1 in the presence of *S. aureus*, and even slightly increased the induction of TAP during *S. aureus* infection. Finally, this strain did not alter the expression of the pattern recognition receptors TLR2 and NOD2. This study supports that *L. casei* BL23 displayed anti-inflammatory properties in a mastitis context. These results open the way to further characterization of the BL23 probiotic potential in a mastitis context and to a better understanding of how all these immunomodulatory properties combine *in vivo* against mastitis pathogens.

Introduction

Probiotics have been considered as a promising alternative to allopathy in the control of various diseases in humans [1, 2]. This strategy has also been used for the maintenance of animal health and is regarded as a means to reduce massive antibiotic use against infectious diseases, including ruminant mastitis [3-7]. Mastitis is an inflammation of the mammary gland. It is generally due to a bacterial infection, with *Staphylococcus aureus* as one of the main pathogens involved in this disease [8]. Mastitis is responsible for great economic losses in dairy farms and preventive strategies and curative ones, which mainly rely on antibiotic therapies, are not totally effective, prompting the need for alternative or complementary strategies [9].

Probiotic bacteria most commonly used and well documented belong to the group of lactic acid bacteria (LAB), particularly *Lactobacillus* and *Bifidobacterium* genera [10]. Their beneficial effects include a combination of several properties allowing them to combat pathogenic bacteria: competitive adhesion to the epithelial mucosa, secretion of antimicrobial substances, modulation of virulence expression in pathogens and modulation of the host immune system [11, 12]. Beneficial properties of LAB have been explored in the mastitis context, showing promising results [3, 5]. In particular, we have shown that several *Lactobacillus casei* strains, including a strain isolated from the mammary gland and the well-characterized probiotic strain *L. casei* BL23 [13], were able to impair adhesion and internalization of *S. aureus* into bovine mammary epithelial cells (bMEC) [14]. Probiotic potential of *Lactobacillus casei* BL23 and, in particular, its ability to modulate the immune system has been widely investigated in a human intestinal environment [15-17] but is poorly explored in a mastitis context. Only recently was reported the reduction of IL-8 secretion by *Escherichia coli*-stimulated cells in the presence of this strain [7].

During the early stages of mammary gland infection, pathogens are sensed by epithelial cells through the interaction of microbe-associated molecular patterns (MAMP) with pattern recognition receptor (PRR) such as Toll-like Receptor (TLR2) and Nucleotide-binding Oligomerization Domain proteins (NOD1 and NOD2). This results in the secretion of cytokines and subsequent recruitment of blood leukocytes, such as neutrophils at the site of infection [18]. The response of mammary gland is notably characterized by the synthesis of proinflammatory cytokines such as IL6, IL-1β, TNF-α and IL8. The first three cytokines play an important role in the acute-phase response, TNF-α and IL-1β are notably involved in the stimulation of neutrophil bactericidal activity [19, 20]. IL-8 is an important chemokine that recruits neutrophils to the infection site [21]. In addition, many antimicrobial peptides have also been identified in the bovine mammary gland such as tracheal antimicrobial peptide (TAP), lingual antimicrobial peptide (LAP) and β-defensin 5 [22, 23].

This study aimed to further investigate the impact of *L. casei* BL23 on the innate immune response in a mastitis context, by determining the effect of this strain on the expression of the above mentioned PRR, interleukins and defensins by bMEC PS line either alone or in the presence of *S. aureus*. We showed that *L. casei* BL23 was able to modulate the expression of several interleukins, whereas it hardly affected the expression of PRR or defensins in the conditions used. Implications of these modulations are discussed.

Material and Methods

Bacterial strains and culture conditions

Lactobacillus casei BL23 [13] and *S. aureus* Newbould 305 (hereafter referred to as N305) were used for immunomodulation tests. Subculture of *S. aureus* was grown in brain-heart infusion medium (BHI; pH 7.4; BD, Le Pont de Claix, France) at 37°C under agitation (180 rpm), *L. casei* BL23 was subcultured in Man Rogosa Sharpe medium (MRS; pH 6.8; BD) at 37°C without shaking. Cultures were washed once with phosphate-buffered saline (PBS) and suspended at different concentrations in DMEM for internalization assays.

Bacterial population was achieved using spectrophotometric measurements at 600 nm with a VWR V-1200 spectrophotometer and confirmed by micromethod, as previously described [Baron et al., 2006]. The *S. aureus* population (in CFU/mL) was determined on mannitol salt agar (MSA; D. Dutscher) after 24 h of incubation at 37°C whereas the *L. casei* population was obtained on MRS after 48 h of incubation at 37°C in an anaerobic jar

Mammary epithelial cell culture

The PS cell line (Roussel et al 2015) was cultured in DMEM/F12 advanced medium (D. Dutcher) containing 10 ng/mL of IGF-1 (Peprotech, Rocky Hill, USA), 5 ng/mL of FGF (Peprotech), 5 ng/mL of EGF (Sigma-Aldrich, Saint Louis, USA), 1 µg/mL of hydrocortisone (Sigma-Aldrich), 20 mM of Hepes buffer (D. Dutcher) and 2 mM of glutamine (Gibco, Waltham, USA). Cells were seeded in a 12-well plate and incubated at 37°C in a humidified incubator with 5% carbon dioxide (CO_2). They were cultured for 72 hours to achieve a confluent monolayer (2.5 x 10^5 cells/well). PS cells were washed once with Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) and were cultured for 16 h in fresh stimulation medium (growth medium without growth factors).

Immunomodulation assays

Confluent monolayers of PS cells at 2.5 x 10^5 cells /well were washed twice with HBSS and incubated with 1 mL of *L. casei* BL23 and or *S. aureus* N305 suspended in DMEM. *L. casei* BL 23 was used with a multiplicity of infection (MOI) of 10:1, 100:1 or 1000:1 and *S. aureus* N305 at a MOI of 10:1 or 100:1.

For co-incubation assays, PS cells were incubated with either *L. casei* alone, *S. aureus* alone or both bacteria for two hours at 37°C and 5% CO_2. Cells were washed three times with HBSS and incubated with stimulation medium containing 100 µg/mL gentamicin (Pan Biotech, Aidenbach, Germany) for two hours to kill adhered extracellular bacteria. They were washed three times with HBSS and incubated with stimulation medium containing 25 µg/mL gentamicin for four or twenty additional hours to complete a total period of 8 or 24 hours of incubation, respectively.

For LAB post-treatment assays, cells were incubated with *S. aureus* exactly as described above. 24h post incubation, cells were washed three times with HBSS and incubated with *L. casei* for 2h at 37°C and 5% CO_2. Cells were washed three times with HBSS and incubated with stimulation medium containing 100 µg/mL gentamicin for two hours to kill adhered extracellular bacteria. They were washed three times with HBSS and incubated with stimulation medium containing 25 µg/mL gentamicin for four additional hours to complete a total period of 8 hours of incubation.

After the incubation period, cells were washed one time with HBSS and lysed with RLT buffer of RNeasy Mini Kit (Qiagen, Hilden, Germany) for subsequent RNA extraction.

RNA extraction and reverse transcription qPCR

Total RNA was isolated from PS cells using Rneasy Mini Kit (Qiagen) according to the manufacturer's recommendations. Elimination of contaminating DNA was performed using

DNA-free™ DNase Removal Treatment and Reagents kit (Life Technologies, Waltham, USA). 1 µg DNAse-treated RNA was used for reverse transcription using qScript™ cDNA Synthesis Kit (Quanta Biosciences, Beverly, USA), according to the manufacturer's recommendations.

Quantitative real-time PCR was performed using a CFX96 ™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). The reaction mixture contained SsoAdvanced Universal SYBR® Green Supermix (1x, Bio-Rad), each primer (0.5 µM, see Table 1 for primer sequences) and a cDNA template. Thermal cycling consisted of 30 s at 98°C, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. The genes tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), ribosomal protein L19 (RPL19) and peptidylprolyl isomerase A (PPIA) have been selected to normalization.

Table 1. Oligonucleotide primers used in this study.

Primer	Sequence	Reference
PPIA _FOR	5'-ATGGCAAGACCAGCAAGAAGAA-3'	Deplanche et al., 2016[24]
PPIA_REV	5'-CTTGGAGGGGGATAAGGAAA-3'	
RPL19_FOR	5'-TACTGCCAATGCTCGAATGC-3'	Deplanche et al., 2016[24]
RPL19_REV	5'-TGATACATGTGGCGGTCAATC-3'	
YWHA_FOR	5'-GTAGGAGCCCGTAGGTCATC-3'	This study
YWHA_REV	5'-GCTTGTGAAGCGTTGGGGAT-3'	
IL-6_FOR	5'-TGCTGGTCTTCTGGAGTATC-3'	Bougarn et al., 2011[25]
IL-6_REV	5'-GTGGCTGGAGTGGTTATTAG-3'	
IL-8_FOR	5'-TGGGCCACACTGTGAAAAT-3'	Deplanche et al., 2016[24]
IL-8_REV	5'-TCATGGATCTTGCTTCTCAGC-3'	
TNF-α_FOR	5'-TCTTCTCAAGCCTCAAGTAACAAGC-3'	Bougarn et al., 2011[25]
TNF-α_REV	5'-CCATGAGGGCATTGGCATAC-3'	

IL-1α_FOR	5’-CTGAAGAAGAGACGGTTGAG-3’	Bougarn et al., 2011[25]
IL-1α_REV	5’-ATGCATTCCTGG TGGATGAC-3’	
IL-1β_FOR	5’-CTCTCACAGGAAATGAACCGAG-3’	Bougarn et al., 2011[25]
IL-1β_REV	5’-GCTGCAGGGTGGGCGTATCACC-3’	
NFκB_FOR	5’-ACCTGGGGATCCAGTGTGTA-3’	This study
NFκB_REV	5’-CGCTGCTCTTCTATGGGAAC-3’	
NOD2_FOR	5’-CCCAGGGGCTCAGAACTAACA-3’	Porcherie et al., 2012[26]
NOD2_REV	5’-CCTTCATCCTGGACGTGGTTC-3’	
TAP_FOR	5’-GTAGGAAATCCTGTAAGCTGTG-3’	Bougarn et al., 2011[25]
TAP_REV	5’-GTGTCTTGGCCTTCTTTTAC-3’	
LAP_FOR	5’-TGCTCCTTGCGCTCCTCTTC-3’	Bougarn et al., 2011[25]
LAP_REV	5’-CTCCGAGACAGGTGCCAATC-3’	
DEFβ1_FOR	5’-CTTCTCTTCCTGGTACTGTCT-3’	Bougarn et al., 2011[25]
DEFβ1_REV	5’-GGCGTGAAACAGGTGCCA ATC-3’	

Statistical analysis

Statistical analyses were performed using R software (R development Core Team. R: A Language and Environment for Statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013. Available: http://www.R-project.org). Differences between treatments were assessed by one-way ANOVA, followed by Tukey's Multiple Comparison Test. Differences between treatments were assessed considering a P value of less than 0.05. Each experiment was conducted in biological triplicate.

Results

***L. casei* BL23 modulates the immune response of PS cells stimulated with *S. aureus* N305**

The potential of *L. casei* BL23 to modulate the innate immune response in PS cells was evaluated either in single incubation assays or in incubation assays with *S. aureus* N305 using different MOIs. In a first set of experiments, the expression of interleukins 6 and 8 (IL-6 and IL-8) as well as Tumor Necrosis Factor Alpha (TNF-α) was measured 8h and 24h post-infection (PI). A global overview of data indicated that PS cell response was more pronounced 8h PI compared to 24h PI (Fig. 1). Additional gene expression was thus measured 8h PI. The targeted genes included additional interleukins (IL-17A, IL-17F, IL-1β, IL-1α), pattern recognition receptors involved in bacterial components recognition, such as Toll-like Receptor 2 (TLR2), Nucleotide-binding Oligomerization Domain proteins (NOD1 and NOD2), the nuclear factor NF-κB, as well as antimicrobial peptides such as Defensin β1 (DFβ1), bovine neutrophil beta-defensin 5 (BNBD5), lingual antimicrobial peptide (LAP), and tracheal antimicrobial peptide (TAP) (Fig 2). Expression of IL-17A, IL-17F, TLR2, NOD1 and BNBD5 was too low in our conditions to be detected (data not shown). Incubation of bMEC with *L. casei* BL23 alone at MOI 10:1, 100:1 and 1,000:1 did not result in any significant changes in expression for all the tested genes, although a small decrease of expression could be noticed as a general trend for most genes (Fig 1 and Fig. 2).

Genes could be divided in three groups depending on their expression profile in the presence of *S. aureus* and *L. casei* BL23. Group 1 includes IL-6, IL-8, TNF-α, IL1-α and IL-1β. Expression of group 1 genes strongly increased 8h PI with *S. aureus* N305 at MOI 10:1, compared to PS cells alone. This increased expression was not observed anymore in the presence of *L. casei* BL23 at MOI 1000:1, whereas a lower *L. casei* MOI had no significant impact (Fig 1A, 1C, 1E, 2A, 2B). A similar trend was observed 8h PI only for IL-6 and TNF-α when bMEC were infected by *S. aureus* at MOI 100:1. On the contrary, *L. casei*, at any

MOI, had no significant effect on the expression of IL-8, IL-1α and IL-1β in bMEC cells infected by *S. aureus* at MOI 100:1. At 24h PI, the expression of IL-6 and IL-8 in bMEC infected by *S. aureus* at MOI 10:1 decreased compared to 8h PI while TNF-α expression remained at a high level. In all cases, *L. casei* had no significant effect on these gene expressions at 24h PI.

Group 2 genes include NOD2 and NF-κB, whose expression remains constant in all the tested conditions.

Finally, group 3 genes include antimicrobial peptides TAP, LAP and DFβ1. Their expression was strongly induced in bMEC infected by *S. aureus* at MOI 100:1 either alone or in the presence of *L. casei* BL23 at MOI 10:1, 100:1 and 1,000:1.

Post treatment by *L. casei* BL23 has no effect on *S. aureus*-infected bMEC cells

In order to further investigate the impact of *L. casei* BL23 on the innate immune response of bMEC, post treatment by *L. casei* BL23 of *S. aureus*-infected cells was performed. bMEC were incubated with *L. casei* 24h post-infection by *S. aureus*. Impact of post-treatment by *L. casei* on the expression of IL-6, IL-8 and TNF-α was monitored 8h post incubation by *L. casei*. None of the post-treatment tested significantly modified the expression of IL-6, IL-8 and TNF-α (Fig 3).

Discussion

Probiotic properties of LAB have been widely explored for humans, especially against the inflammatory diseases of the intestinal tracts, leading to the successful development of probiotic solutions [11, 27]. Health benefits include prevention or mitigation of IBDs, IBS and ulcerative colitis in humans and the reduction of symptoms of constipation and diarrhea [15, 16, 28-33]. Among the bacteria which have been deeply investigated, some LAB strains exerting beneficial properties have emerged as reference strains. This is the case for *L. casei* BL23. In a mouse model, this strain was previously shown to confer anti-inflammatory effects in a dextran sulfate sodium (DSS) mouse model of UC [15, 16]. This strain was also demonstrated to display protective effects on anti-colorectal cancer [17]. We recently started to explore the beneficial properties of *L. casei* BL23 in another context, i.e. the bovine mastitis context and we observed promising properties. *L. casei* BL23 was shown to colonize bMEC and to inhibit internalization of *S. aureus* into bMEC [14]. In the present study, we further explored the properties of this strain in a mastitis context and established that this strain was able to modulate the innate immune response of bMEC or *S. aureus*-stimulated bMEC. Hence, *L. casei* BL23 was found to decrease the expression of several pro-inflammatory cytokines, including IL-6, IL-8, IL-1α, IL-1β and TNF-α, in *S. aureus*-stimulated bMEC. On the contrary, *L. casei* did not affect the expression of neither PRR NOD2 nor nuclear factor NF-κB. Likewise, the expression of defensins was not modulated by the presence of *L. casei*, except for TAP whose induction by *S. aureus* at MOI 100:1 was more pronounced (~ 2-fold) in the presence of *L. casei*.

First, it should be mentioned here that the immunomodulatory properties of *L. casei* BL23 were investigated using a newly described cell lines called PS [34]. This cell line was shown to be more responsive than the usually studied MAC-T cell line to bacterial infection in terms of interleukin production. The inflammatory response of PS line has been mainly studied in

response to *E. coli* infection, and until recently [24], no data were available on the impact of *S. aureus* on its innate immune response. Here we showed that infection by *S. aureus* resulted in the induction of the pro-inflammatory interleukins IL-6, IL-8, TNF-α, IL-1α and IL-1β, which is in agreement with previous data obtained in vivo with *S. aureus* MAMPS [35] or in vitro using primary cell cultures [25, 36] or bMEC cell line [37]. We also observed induction of the expression of several defensins, including TAP, LAP and DEFB1, for a *S. aureus* MOI of 100:1. Induction of these antibacterial peptides has been previously reported during infection [38]. Fu et al. also reported induction of the BNBD5 defensin by *S. aureus*. However, the expression of the BNBD5 encoding gene remained below the detection threshold in our study [39]. Contrary to previous reports [35, 36], we did not observe any induction of TLR2 expression by *S. aureus*. TLR2 has been identified as PRR for peptidoglycans and lipoteichoic acids from Gram-positive bacteria and is involved in the initiation of the innate immune response [40, 41]. However, TLR2 induction is transient and occurs early after infection (3h) [36], which could explain this discrepancy as we monitored the innate immune response later, at 8h PI. Altogether, these results suggest that the PS cell line response to *S. aureus* infection is closed to the one observed in vivo or using bMEC primary culture and that this line can be used as a proxy to decipher the impact of *L. casei* in a mastitis context and more specifically during *S. aureus* infection.

As a whole, our results support that *L. casei* BL23 displayed anti-inflammatory properties in a mastitis context, which is in agreement with previous studies on this strain in human gut context. This strain was shown to decrease the quantities of pro-inflammatory cytokines such as IL-6 and IL-1α in a DSS-induced murine model of ulcerative colitis [16]. Likewise, this strain displayed anti-inflammatory properties in a 1,2dimethylhydrazine (DMH)-associated colorectal cancer model as revealed by a decrease of TNF-α in the intestinal content of *L. casei*-treated mice and a decrease of IL-6 in colon samples [17]. Apart from these in vivo

studies, *L. casei* BL23 revealed an anti-inflammatory profile when applied to PBMC, resulting in a high ratio between the anti-inflammatory interleukin IL-10 and the pro-inflammatory interleukin IL-12 [42]. Of note, we had previously reported the capacity of *L. casei* BL23 to decrease IL-8 production by *E. coli*-stimulated bMEC [7], suggesting that anti-inflammatory properties of *L. casei* BL23 in mastitis context is not restricted to one pathogen but could be a general property. The anti-inflammatory profile is observed mainly during co-incubation with *S. aureus*. A similar trend was obtained on interleukin expression when *L. casei* was incubated with bMEC alone albeit the effect was not statistically significant. It should be noticed here that the capacity of *L. casei* BL23 to decrease several pro-inflammatory interleukins depends on the MOI used for both strains. Anti-inflammatory properties of *L. casei* BL23 were observed for the highest *L. casei* MOI only. Besides, the decrease of pro-inflammatory IL expression was observed for all the pro-inflammatory IL at 8 h PI for a *S. aureus* MOI of 10:1, whereas it was significant only for IL-6 at a *S. aureus* MOI of 100:1. Indeed the expression level of the other IL, IL-8, IL-1α and IL-1β, was hardly affected at a *S. aureus* MOI of 100:1 compared to bMEC alone. MOI-dependent modulation of interleukin expression has already been reported, underlying the fine-tuned regulation of the innate immune response [43]. Likewise, the impact of *L. casei* BL23 is time-dependent. No significant effect of *L. casei* BL23 was observed 24h PI for IL-6 and IL-8, in relation with a low expression of these IL even in the presence of *S. aureus* alone compared to bMEC control. Such transient induction of IL-6 and IL-8 expression by *S. aureus* has already been reported [24]. In accordance, post-treatment of *S. aureus*-stimulated bMEC by *L. casei* BL23 did not significantly modulate expression of these interleukins.

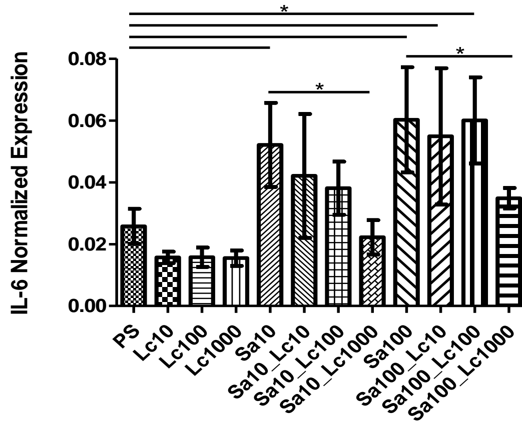
Apart from the impact on pro-inflammatory interleukins, *L. casei* did not alter the expression of the other genes of the innate immune response tested in this study. Hence, the expression of PRR such as TLR2 and NOD2, involved in the recognition of bacteria through interaction

with lipoteichoic acid and peptidoglycan fragments respectively [44, 45] remained unchanged in all the tested conditions. Likewise, the expression of NF-kB was affected neither by the presence of *L. casei* nor that of *S. aureus*. Nevertheless, this nuclear factor is mainly controlled at the post-transcriptional level rather than at the transcriptional level. The impact of *L. casei* BL23 on the activity of this pivotal regulator of the innate immune response will deserve further investigations, all the more that the expression of some NF-kB targets, such as IL-1β, IL-6 and TNF-α is modulated. Nevertheless, induction of IL-1β, IL-6, TNF-α and IL-8 in a NF-kB-independent manner has also been reported in response to *S. aureus*-infection [36]. At last, *L. casei* BL23 did not significantly modulate the expression of most defensins which were induced by *S. aureus* at an MOI of 100:1, except for TAP, whose induction was even more pronounced in the presence of *L. casei*. This indicates that *L. casei* exerts either a neutral or positive effect on the induction of these antimicrobial peptides.

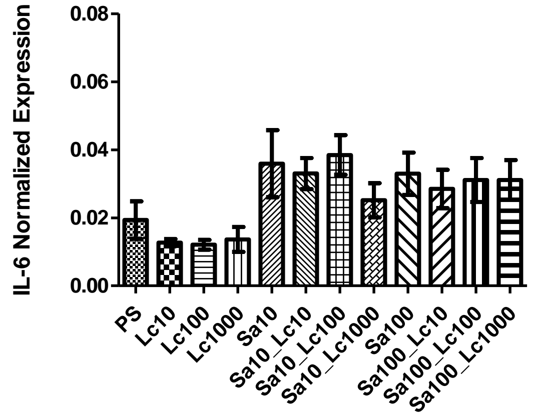
In conclusion, our study revealed anti-inflammatory properties of *L. casei* BL23 when incubated on *S. aureus*-stimulated bMECs, indicating that this strain displays similar immunomodulatory potential in the mammary as well as in the gut contexts. Among the pro-inflammatory IL whose expression was lower in the presence of *L. casei* BL23, TNF-α, IL-1β and IL-6 play an important role in the acute phase response of inflammation. TNF-α is able to stimulate the release of various inflammatory mediators such as NO and oxygen-free radicals by endothelial cells and leukocytes, thereby promoting neutrophil phagocytosis [46]. IL-1β activates synthesis of acute phase proteins, attracts aggregates of neutrophils, and stimulates immune cells and endothelial cells to produce various inflammatory cytokines and chemokines [47, 48]. Both TNF-α and IL-1β can cause fever. Considering the deleterious effects of these interleukin inductions, a lower induction of these interleukins by *L. casei* BL23 may reduce tissue damages. This could be of particular interest during acute infections such as those generally resulting from *E. coli* infections. Nevertheless, attenuation of

inflammation by *L. casei* and especially the lower induction of IL-8 may also have adverse effects on the pathogen clearance in case of *S. aureus* mastitis, which does not induce a strong cytokine response during mammary infection [24]. IL-8 is an important chemokine that recruits neutrophils to the infection site. A lower secretion of IL-8 may result in a lower recruitment of neutrophils to the infection sites and, as a consequence, may preclude from efficient pathogen clearance. Interestingly, although *L. casei* BL23 displayed anti-inflammatory properties, it exerted a neutral or positive effect on defensin expression. These results, combined to our previous results revealing the capacity of *L. casei* BL23 to impair *S. aureus* internalization, invite us to further explore the impact of this strain and other LAB in a mastitis context and to investigate the resulting impact of the above mentioned properties in vivo.

A



B



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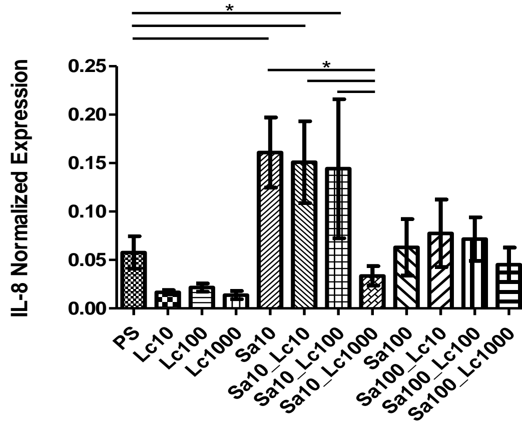

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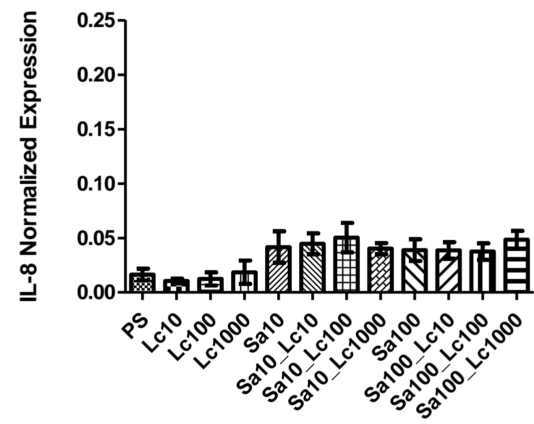

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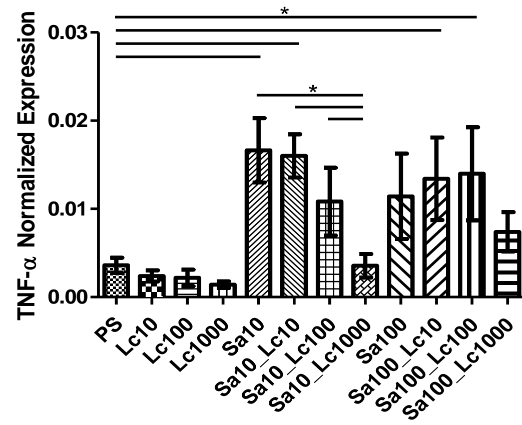

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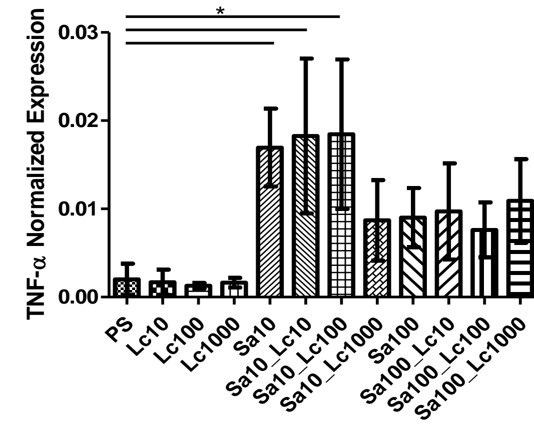
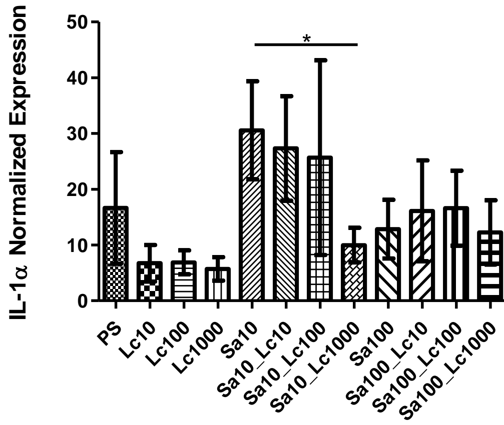


Figure 1. Impact of *L. casei* BL23 on IL6 (A, B), IL8 (C, D), TNF-α (E, F) expression by bMEC (PS line) during infections by *S. aureus* N305. bMEC were infected with *S. aureus* N305 at MOI 10:1 and 100:1 (Sa10 or Sa100), either alone or in the presence of *L. casei* BL23 at MOI 10:1, 100:1 and 1,000:1 (Lc10, Lc100 or Lc1000). Expression was measured 8h post-infection (A, C, E) or 24h post-infection (B, D, F). Expression was determined as normalized expression with regard to 3 control genes (see material and methods for details). Each experiment was done in triplicate, and differences between groups were compared using one-way ANOVA with Tukey's Multiple Comparison Test. *: $P < 0.05$. Significant differences of expression with regard to PS cells alone are indicated as far as significant differences between *S. aureus* infected cells at various *L. casei* BL23 MOI.

A



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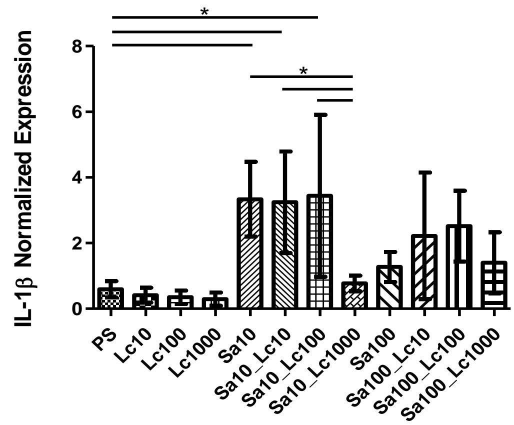

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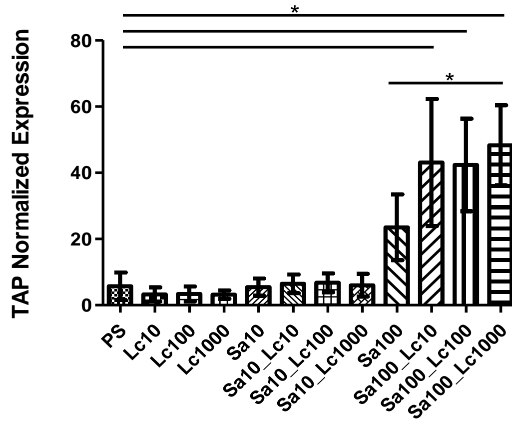

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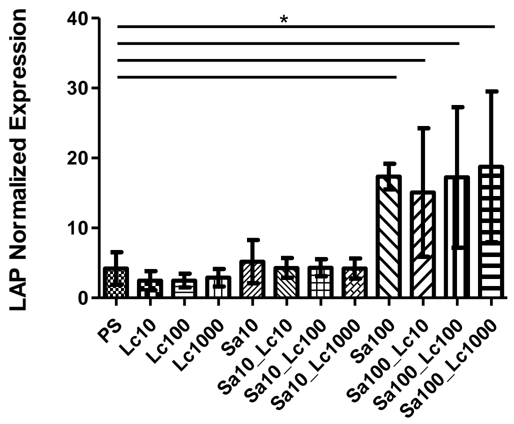

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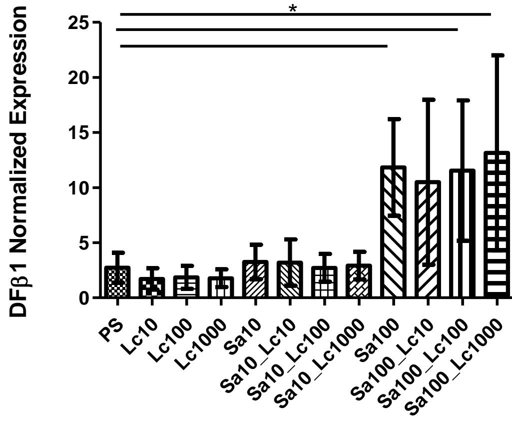

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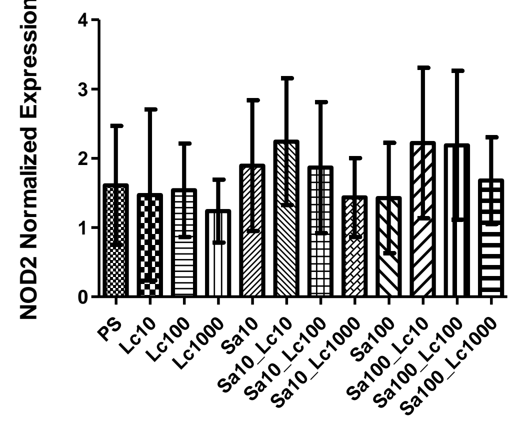

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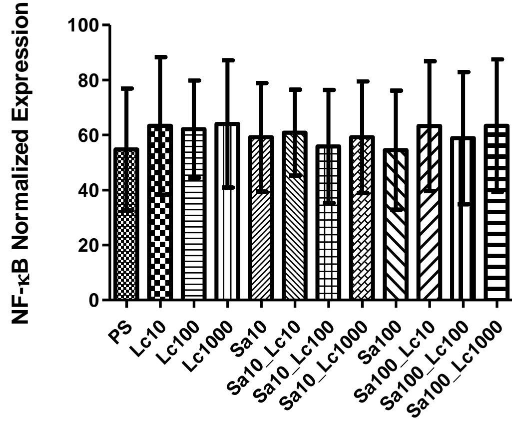

Figure 2. Impact of *L. casei* BL23 on IL-1α (A), IL-1β (B), TAP (C), LAP (D), DFβ1 (E), NOD2 (F), NF-κB (G) expression by bMEC (PS line) during infections by *S. aureus* N305. bMEC were infected with *S. aureus* N305 at MOI 10:1 and 100:1 (Sa10 or Sa100), either alone or in the presence of *L. casei* BL23 at MOI 10:1, 100:1 and 1,000:1 (Lc10, Lc100 or Lc1000). Expression was measured 8h post-infection. Expression is expressed as 1000x the normalized expression with regard to 3 control genes (see material and methods for details). Each experiment was done in triplicate, and differences between groups were compared using one-way ANOVA with Tukey's Multiple Comparison Test. *: $P < 0.05$. Significant differences of expression with regard to PS cells alone are indicated as far as significant differences between *S. aureus* infected cells at various *L. casei* BL23 MOI.

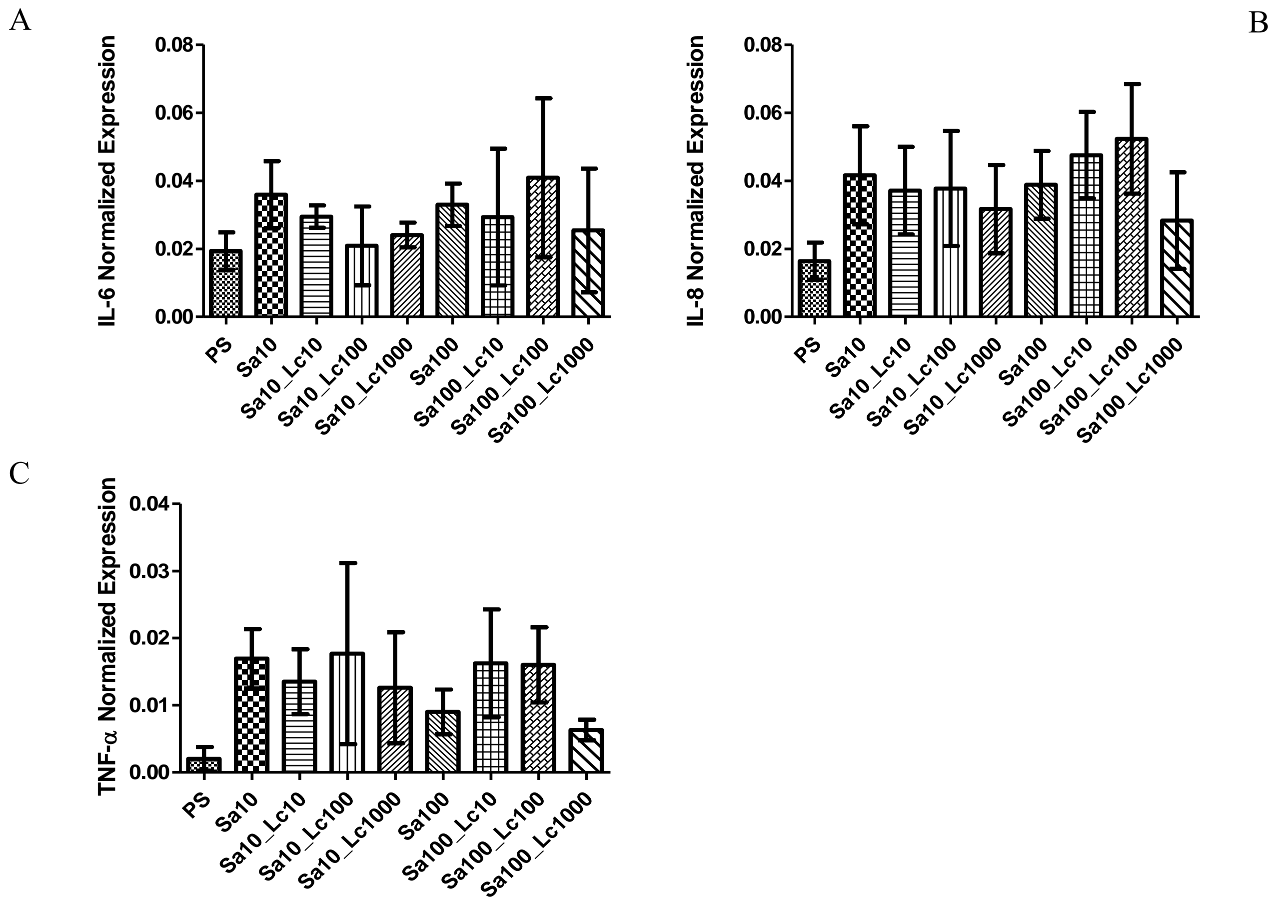

Figure 3. Impact of *L. casei* BL23 on IL6 (A), IL8 (B), TNF-α (C) expression by bMEC (PS line) following infections by *S. aureus* N305. bMEC were infected with *S. aureus* N305 at MOI 10:1 and 100:1 (Sa10 or Sa100). 24h post-infection by *S. aureus*, cells were further incubated with *L. casei* BL23 at MOI 10:1, 100:1 and 1,000:1 (Lc10, Lc100 or Lc1000). Expression was measured 8h post-incubation by *L. casei* BL23. Expression was determined as normalized expression with regard to 3 control genes (see materials and methods for details). Each experiment was done in triplicate, and differences between groups were compared using one-way ANOVA with Tukey's Multiple Comparison Test. *: $P < 0.05$.

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CONCLUSÕES E PERSPECTIVAS

A mastite representa uma das principais causas de perdas econômicas na indústria de lacticínios (Oviedo-Boyso et al., 2007). Até o momento, a prevenção por meio de vacinação ou o tratamento por antibioticoterapia se mostraram pouco eficazes (Barkema et al., 2006 ; Pereira et al., 2011; Wallemacq et al., 2010). Adicionalmente, a antibioticoterapia resulta em risco de emergência e propagação de linhagens bacterianas resistentes no meio ambiente (White e McDermott, 2001). Diante disso, diversos estudos têm buscado o desenvolvimento de métodos alternativos eficientes e mais seguros para o controle dessa doença (Varshney e Naresh, 2005; Peton e Le Loir, 2014; Mullen et al., 2014; Gomes and Herinques, 2016).

A utilização de bactérias lácticas probióticas parace ser uma estratégia promissora para o controle da mastite. Uma equipe irlandesa mostrou a eficácia de uma linhagem de *L. lactis* na prevenção e até mesmo no tratamento da mastite bovina, apesar dos mecanismos responsáveis pelos efeitos benéficos não estarem completamente elucidados (Klostermann et al., 2008; Crispie et al., 2008). A diversidade desses mecanismos pode explicar a nossa compreensão parcial desses efeitos e do papel dos fatores bacterianos durante as complexas interações entre probiótico-patógeno-hospedeiro.

No contexto da mastite, uma das hipóteses levantadas para explicar a persistência de infecções desencadeadas por *S. aureus* envolve o processo de invasão (adesão e internalização) da bactéria nas células da glândula mamária (Sinha e Fraunholz, 2010; Fraunholz e Sinha, 2012). A fim de avaliar o potencial probiótico de BL nesse contexto, para desenvolver uma método de controle alternativo, nossa equipe testou a capacidade de bactérias lácticas inibirem essas etapas chaves do ciclo infeccioso de *S. aureus* sobre um modelo *in vitro* de células epiteliais mamária bovinas (CEMb). Entre as várias linhagens, nós utilizamos *L. casei* BL23, uma linhagem com efeitos probióticos já descritos em outros contextos e com genoma sequenciado disponível. Esse estudo permitiu demonstrar o potencial inibitório de *L. casei* BL23 sobre a internalização de *S. aureus* em CEMb (Bouchard et al., 2013). No entanto, os mecanimos subjacentes a esse antagonismo não foram determinados, fato que fomentou o desenvolvimento desse trabalho.

A hipótese do presente projeto baseou-se na participação das enzimas sortases de *L. casei* BL23 nesse antagonismo. Essas enzimas estão implicadas no ancoramento de proteínas com motivo LPXTG na parede celular bacteriana. As proteínas ancoradas à superfície bacteriana são importantes moléculas para a interação bactéria-hospedeiro, favorecendo a colonização bacteriana através de processos tais como a adesão e autoagregação (Mokrozub et al., 2015). A análise do genoma de *L. casei* BL23 revelou a presença de 23 genes que codificam proteínas que possuem o motivo LPXTG na região C-terminal. Entre essas

proteínas, oito adesinas possuem características que as permitem entrar em competição com as adesinas de *S. aureus* pelos receptores celulares (Muñoz-Provencio et al., 2012).

Com a finalidade de verificar nossa hipótese, a estratégia adotada foi a utilização de mutantes sortases para avaliar o impacto da interrupção nos genes sortases (srtA1, srtA2, srtC1 e srtC2) sobre a capacidade de inibição de *L. casei* BL23 sobre a adesão e internalização de *S. aureus* nas CEMb. Isso permitiu direcionar as seguintes questões:

1. *L. casei* BL23 conserva a capacidade de inibir a internalização de *S. aureus* em células mamárias bovinas?
2. Qual o perfil de proteínas de superfície da parede celular de *L. casei* BL23 selvagem e dos mutantes sortases?
3. *L. casei* BL23 tem capacidade de modular a resposta imune do hospedeiro?

Nessas últimas páginas do manuscrito, nós iremos discutir os resultados obtidos ao longo desse trabalho. E por fim, as perspectivas desse projeto serão apresentadas.

- **Impacto das mutações sortases sobre a capacidade de *L. casei* BL23 inibir a internalização de *S. aureus* em células epiteliais mamárias bovinas**

Os nossos resultados mostraram que *L. casei* BL23 inibe 58% da internalização de *S. aureus* em CEMb. Esta capacidade de inibição é significativa quando BL23 é usada com uma multiplicidade de infecção de 2,000:1. Deve-se notar que *L. casei* BL23 também apresenta uma capacidade de internalização nas CEMb com esse MOI. No entanto, esta capacidade de internalização é baixa em comparação com *S. aureus*, apesar da multiplicidade de infecção ter sido vinte vezes maior do que a adotada para *S. aureus* (MOI 2,000:1 vs 100:1). Enquanto *S. aureus* nesse estudo teve uma internalização média de 2 bactérias por célula CEMb, *L. casei* BL23 apresentou uma internalização média de uma bactéria a cada 20 células.

Nós verificamos em seguida, se a ausência das enzimas sortases influenciam no potencial inibitório de *L. casei* BL23 frente a invasão de *S. aureus*. Para todos os mutantes sortases de *L. casei* BL23, nós mostramos uma redução da capacidade de sua atividade inibitória contra *S. aureus*. O mutante srtA2 foi a linhagem mutante que apresentou a perda mais expressiva da inibição. Em adição, srtA2 favoreceu uma maior taxa de internalização de *S. aureus* comparada à condição controle. Esses resultados permitem identificar um novo fator genético implicado na interação entre *L. casei*-*S. aureus*-bMEC. No entanto, vários pontos ainda devem igualmente ser considerados a fim de confirmar a sua confiabilidade.

A análise da complementação do mutante srtA2 por um gene srtA2 intacto deve ser realizada com o intuito de confirmar que o fenótipo observado é de exclusiva dependência da ausência de atividade de sortase A2, visto que os demais mutantes sortases (srtA1, srtC1, srtC2) também demonstraram em menor grau uma alteração na capacidade inibitória.

O gene srtA2 está presente apenas em algumas linhagens de *L. casei* (Muñoz-Provencio et al., 2012). Uma alternativa interessante para confirmar sua importância consistiria em determinar se a presença e o nível de expressão de srtA2 estão correlacionados à atividade inibitória em outras linhagens de *L. casei* portando ou não srtA2. Além disso, a verificação da expressão das demais sortases de *L. casei* BL23 em todos os mutantes sortases é importante para determinar se há algum tipo de processo compensatório devido à ausência da atividade de ancoramento de proteínas na superfície celular por SrtA2. Isso permitiria verificar se as sortases têm uma especificidade pelos substratos ou se elas podem apoiar um painel mais amplo de proteínas LPXTG em *L. casei* BL23.

- **Impacto das mutações sortases sobre o perfil das proteínas da parede celular de *L. casei* BL23**

Para um sistema biológico, a comunicação com o ambiente é mediada por quase todas as classes de moléculas biológicas. Contudo, as proteínas expostas a superfície bacteriana configuram a principal categoria devido à diversidade de sua estrutura, função e localização. Elas atuam principalmente no transporte de nutrientes ou íons, atividade enzimática e como fatores de ligação a superfícies biológicas (Olaya-Abril et al., 2013). O estudo proteômico através da espectometria de massa representa uma poderosa e efetiva abordagem para identificar as proteínas potencialmente implicadas nesse processo de interação bactéria-hospedeiro (Yang et al., 2015).

Nesse estudo, o impacto da interrupção de srtA2 sobre a superfície celular de *L. casei* BL23 foi determinado através de *shaving* enzimático. Essa técnica é de fácil aplicação, mas pode gerar resultados com um certo viés devido à presença de proteínas citoplasmáticas identificadas nas amostras, apesar da aparente ausência de lise celular. Essas proteínas não podem ser sistematicamente descartadas das análises, uma vez que numerosas proteínas preditas como citoplasmáticas tem sido identificadas sobre a superfície bacteriana durante trabalhos anteriores. Embora, os mecanismos de endereçamento dessas proteínas sejam ainda mal conhecidos, elas podem desempenhar um papel fisiológico na superfície (por exemplo,

adesão à células dos hospedeiro) diferentes das suas funções citoplasmáticas. Por essas características, elas são denominadas *moonlighting proteins*.

O perfil de proteínas de superfície foi estudado com as linhagens BL23 selvagem e srtA2 com a finalidade de identificar proteínas diferencialmente produzidas e potencialmente associadas aos fenótipos observados, especialmente à capacidade inibitória de *L. casei* BL23. Em paralelo, nós observamos o envelope bacteriano dessas linhagens por microscopia eletrônica de transmissão a fim de avaliar o impacto da mutação srtA2 sobre a arquitetura da parede. Nós assim observamos uma redução da espessura da parede celular bacteriana que poderia contribuir para uma menor resistência do mutante srtA2 ao estresse oxidativo. A integridade e a estabilidade do peptidoglicano são importantes para tolerar os estresses relacionados aos fatores ambientais (Chapot-Chartier e Kulakauskas, 2014).

As análises dos dados do *shaving* permitiram a seleção de 26 proteínas diferencialmente presentes na superfície bacteriana, e apenas 2, InlJ e BnaG possuem o motivo LPXTG. Esses resultados abrem pistas para a seleção de alvos para o desenvolvimento futuro de estudos mais específicos. Assim, entre as proteínas identificadas, aquelas que possuem o motivo LPXTG representam os alvos privilegiados. Isso requer a obtenção de mutantes de inativação para os 2 genes codificantes para InlJ e BnaG. A análise fenotípica desses mutantes permitirá aprofundar nosso estudo e determinar se a perda da inibição observada com o mutante BL23 srtA2 é devido diretamente a ausência da atividade srtA2 ou a um efeito indireto ligado à ausência desses 2 substratos, InlJ e/ou BnaG na superfície bacteriana.

- **Impacto de *L. casei* BL23 sobre a modulação da resposta imune inata do hospedeiro**

A modulação da resposta imune é um dos mecanismos utilizados por bactérias probióticas para exercer seus efeitos benéficos sobre o hospedeiro (Amdekar et al., 2011; Lebeer et al., 2010; Tsai et al., 2012). A proteção da glândula mamária passa pela produção de algumas citocinas como IL-6, IL-8, TNF-α, assim como pela expressão de alguns peptídeos antimicrobianos, principalmente TAP e LAP (Rainard et Riollet, 2006; Oviedo-Boyso et al., 2007).

O objetivo dessa etapa foi verificar se *L. casei* BL23 tem a capacidade de modular a resposta imune frente a *S. aureus* em um modelo celular CEMb utilizando a linhagem PS (escolhida pelo fato de responder melhor ao estímulo comparada à linhagem MAC-T).

Esses resultados são ainda preliminares e necessitam de experimentos suplementares. Até o momento, a coincubação entre *L. casei* BL23 e *S. aureus* NB305 em CEMb PS apresentou resultados promissores, ao que concerne à redução de IL-6, IL-8, TNF-α, IL-1 α e IL-1β, importantes marcadores da resposta inflamatória. A condição de pós-incubação com *L. casei* BL23 foi igualmente testada com o intuito de avaliar a capacidade de BL23 tratar infecções causadas por *S. aureus*. No entanto, nenhuma capacidade significativa de modular a resposta imune por *L. casei* BL23 foi encontrada nessa condição.

Os resultados obtidos até o momento consistem de dados preliminares capazes de estabelecer as condições mais adequadas para a avaliação da contribuição de srtA2 de *L. casei* BL23 para a modulação da resposta imune. Além da expressão de genes de citocinas que inicialmente foram avaliadas por PCR quantitativa, também está previsto a dosagem por ELISA para confirmar os resultados. Outras citocinas além daquelas testadas aqui poderão igualmente ser pesquisadas (por exemplo, IL-10 ou IL-32).

Outro fato importante a ser considerado é que o sistema imune do hospedeiro responde de maneiras diferentes conforme o tipo de patógeno a que ele é exposto. Em particular, as infecções intramamárias provocadas por *S. aureus* e *E. coli* apresentam perfis diferentes. A infecção causada por *E. coli* tem um perfil agudo, podendo ser eliminada em poucos dias. Por outro lado, a infecção por *S. aureus* provoca uma resposta inflamatória geralmente mais fraca mas frequentemente resulta em uma infecção crônica, talvez devido a essa reação inflamatória moderada (Bannerman et al., 2004). Diante disso, é importante observar o tipo de resposta imune no nosso modelo estimulado por outros importantes patógenos, como *E. coli.*

Por fim, os probióticos são mais frequentemente considerados em um contexto de prevenção do que tratamento. Nós iremos avaliar o impacto de um pré-tratamento com *L. casei* BL23 sobre a resposta inflamatória das CEMb durante uma infecção por *S. aureus*.

- **Conclusão final**

Os resultados obtidos nessa tese são o primeiro passo para a compreensão dos mecanismos utilizados por *L. casei* BL23 para inibir a internalização de *S. aureus* em células epiteliais mamárias bovinas. O conjunto desses resultados obtidos *in vitro* evidentemente não representam o comportamento global dos probióticos *in vivo*. Mas essas abordagens constituem uma boa ferramenta capaz de triar e identificar as melhores linhagens para a formulação de um probiótico mamário. A possibilidade de desenvolver um tratamento alternativo aos antibióticos, utilizando bactérias probióticas do ecossistema mamário bovino,

pode representar uma alternativa eficaz, segura e menos onerosa, para reduzir os prejuízos associados a mastite, evitar a emergência de patógenos antibioresistentes e assegurar o bem-estar animal.

CONCLUSIONS ET PERSPECTIVES

La mammite constitue l’une des principales pertes économiques pour la filière laitière (Oviedo-Boyso et al., 2007). À ce jour, la prévention par la vaccination ou le traitement avec des antibiotiques se montrent peu voire pas efficaces (Barkema et al., 2006 ; Pereira et al., 2011 ; Wallemacq et al., 2010). De plus, l’antibiothérapie engendre un risque d'émergence et de propagation de souches bactériennes résistantes dans l'environnement (White et McDermott, 2001). Aussi est-il utile de développer des méthodes alternatives efficaces et plus sûres pour le contrôle de cette maladie (Varshney et Naresh, 2005 ; Peton et Le Loir, 2014 ; Mullen et al., 2014 ; Gomes et Herinques, 2016).

L'utilisation de bactéries lactiques (BL) probiotiques peut être une stratégie prometteuse pour le contrôle de la mammite. Une équipe irlandaise a en effet montré l’efficacité d’une souche de *Lactococcus lactis* dans la prévention et même le traitement de mammites bovines, même si les mécanismes responsables des effets bénéfiques ne sont pas complètement élucidés (Klostermann et al., 2008 ; Crispie et al., 2008). La diversité de ces mécanismes peut expliquer notre compréhension incomplète de ces effets et du rôle des facteurs bactériens dans les interactions complexes probiotique-pathogène-hôte.

Dans le contexte de la mammite, l'une des hypothèses pour expliquer la persistance des infections à *S. aureus* implique l'invasion (adhésion et internalisation) des bactéries dans les cellules de la glande mammaire (Sinha et Fraunholz, 2010 ; Fraunholz et Sinha, 2012). Afin d'évaluer le potentiel probiotique des BL dans ce contexte et pour développer une méthode alternative de contrôle, notre équipe a testé la capacité des BL à inhiber ces étapes clés du cycle infectieux de *S. aureus* sur un modèle *in vitro* de cellules épithéliales mammaires bovines (CEMb). Entre autres souches, nous avons utilisé *L. casei* BL23, une souche dont les effets probiotiques sont déjà décrits dans d’autres contextes et dont la séquence génomique est connue. Cette étude a permis de montrer le potentiel d'inhibition de *L. casei* BL23 sur l'internalisation de *S. aureus* aux CEMb (Bouchard et al., 2013). Cependant, les mécanismes sous-jacents à cet antagonisme n'ont pas été déterminés, ce qui a encouragé le développement de ce travail.

Nous avons émis l'hypothèse que les sortases de *L. casei* BL23 sont impliquées dans cet antagonisme. Ces enzymes sont responsables de l'ancrage des protéines portant le motif LPXTG à la paroi bactérienne. Les protéines ancrées à la surface bactérienne sont des molécules importantes pour l'interaction bactérie-hôte, favorisant la colonisation bactérienne par des processus tels que l’adhésion et l’auto-agrégation (Mokrozub et al., 2015). L'analyse du génome de *L. casei* BL23 a révélé la présence de 23 gènes codant pour des protéines ayant un motif LPXTG dans la région C-terminale. Parmi ces protéines, 8 adhésines ont des

caractéristiques qui leur permettraient d'entrer en compétition avec les adhésines de *S. aureus* pour les récepteurs cellulaires (Muñoz-Provencio et al., 2012).

Afin de vérifier notre hypothèse, la stratégie adoptée a été l'utilisation de mutants sortases pour évaluer l'impact des disruptions de gènes sortases (*srtA1*, *srtA2*, *srtC1* et *srtC2*) sur la capacité d'inhibition de *L. casei* BL23 sur l'adhésion et l'internalisation de *S. aureus* dans les CEMb. Ceci a permis d'adresser les questions suivantes :

1. Les mutants sortases de *L. casei* BL23 conservent-ils la capacité à inhiber l'internalisation de *S. aureus* aux CEMb ?
2. Quel est le profil des protéines de surface de *L. casei* BL23 sauvage et des mutants sortases ?
3. *L. casei* BL23 a-t-elle la capacité de moduler la réponse immunitaire de la cellule hôte?

Dans ces quelques dernières pages du manuscrit, nous allons discuter des résultats obtenus au cours de ce travail. Enfin, les perspectives de ce projet seront présentées.

- **Impact des mutations sortases sur la capacité de *L. casei* BL23 à inhiber l'internalisation de *S. aureus* dans les cellules épithéliales mammaires bovines**

Nos résultats ont montré que *L. casei* BL23 inhibe 58% de l'internalisation de *S. aureus* dans les CEMb. Cette capacité d'inhibition est significative quand BL23 est utilisée à une multiplicité d'infection de 2,000: 1. Il est à noter que *L. casei* BL23 présente également une capacité à internaliser dans les CEMb à cette MOI. Toutefois, cette capacité d'internalisation est faible comparée à celle de *S. aureus*, et cela bien que la multiplicité d'infection soit vingt fois plus élevée que celle adoptée pour *S. aureus* (MOI de 2,000:1 versus 100:1). Alors que *S. aureus* dans cette étude avait une internalisation moyenne de 2 bactéries par cellule CEMb, *L. casei* BL23 présente une internalisation moyenne d'une bactérie internalisée pour 20 cellules.

Nous avons ensuite vérifié si l'absence des sortases interfère dans le potentiel inhibiteur de *L. casei* BL23 vis-à-vis de l'invasion de *S. aureus*. Pour tous les mutants sortases de *L. casei* BL23, nous avons montré une réduction de la capacité de leur activité inhibitrice contre *S. aureus*. Le mutant *srtA2* a été celui qui a présenté la plus importante perte d'inhibition. En outre, il a favorisé un taux d'internalisation de *S. aureus* plus élevé par rapport à la condition contrôle. Ce résultat permet d'identifier un nouveau facteur génétique impliqué

dans l'interaction *L. casei*-*S. aureus*-bMEC. Cependant, plusieurs points devraient également être pris en considération afin de confirmer sa fiabilité.

L'analyse de la complémentation du mutant *srtA2* par un gène *srtA2* intact doit être effectuée afin de confirmer que le phénotype observé est dépendant seulement de l'absence d'activité de SrtA2. Les autres mutants sortases (*srtA1, srtC1, srtC2*) ont également montré, dans un moindre degré, une réduction de la capacité inhibitrice.

Le gène *srtA2* est présent uniquement dans certaines souches de *L. casei* (Muñoz-Provencio et al., 2012). Une alternative intéressante pour confirmer l'importance de *srtA2* pourrait consister à déterminer si la présence et le niveau d'expression de *srtA2* sont corrélés à l'activité inhibitrice dans d'autres souches de *L. casei* portant ou non *srtA2*. En outre, la vérification de l'expression d'autres sortases de *L. casei* BL23 dans tous les mutants sortases est importante car la souche pourrait mettre en place des processus de compensation à l'absence d'activité SrtA2 d'ancrage des protéines dans la surface cellulaire. Elle permettrait de vérifier si les sortases ont une spécificité étroite pour leurs substrats ou si elles peuvent prendre en charge un panel plus large de protéines LPXTG chez *L. casei* BL23.

- **Impact des mutations sortases sur le profil des protéines de surface de *L. casei* BL23**

Pour un système biologique, la communication avec l'environnement est réalisée par presque toutes les classes de molécules biologiques. Toutefois, les protéines exposées à la surface bactérienne constituent la principale catégorie en raison de la diversité de leur structure, leur fonction et leur localisation. Elles agissent principalement sur le transport des nutriments et des ions, l'activité enzymatique et comme facteurs de liaison aux surfaces biologiques (Olaya-Abril et al., 2013). L'étude protéomique par spectrométrie de masse est une approche puissante et efficace pour identifier des protéines potentiellement impliquées dans ces processus d'interaction bactéries-hôte (Yang et al., 2015).

Dans cette étude, l'effet de l'interruption de *srtA2* sur la surface des cellules de *L. casei* BL23 a été déterminé par la technique du « shaving » (rasage enzymatique). Cette technique est d'application facile, mais peut produire des résultats avec un certain biais en raison de la présence de protéines cytoplasmiques identifiées dans les échantillons malgré l'absence apparente de lyse cellulaire. Ces protéines ne peuvent pas être systématiquement écartées de l'analyse, car de nombreuses protéines prédites comme cytoplasmiques ont été identifiées sur la surface bactérienne lors de travaux précédents. Bien que les mécanismes d'adressage de ces

protéines soient encore mal connus, elles peuvent jouer un rôle physiologique à la surface (par exemple, adhésion aux cellules de l'hôte) différent de leur rôle intracytoplasmique. A ce titre, elles sont dénommées « moonlighting proteins ».

Les profils de protéines de surface ont été étudiés sur les souches BL23 sauvage et *srtA2* afin d'identifier des protéines différentiellement produites et potentiellement associées aux phénotypes observés, notamment à la capacité d'inhibition de *L. casei* BL23. En parallèle, nous avons observé l'enveloppe bactérienne de ces souches par microscopie électronique à transmission pour évaluer l'impact de la mutation *srtA2* sur l'architecture de la paroi. Nous avons ainsi observé une réduction de l'épaisseur de la paroi cellulaire bactérienne qui pourrait contribuer à la moindre résistance du mutant *srtA2* au stress oxydatif. L'intégrité et la stabilité du peptidoglycane sont en effet importantes pour tolérer les stress liés à des facteurs environnementaux (Chapot-Chartier et Kulakauskas, 2014).

L'analyse des données de shaving a permis la sélection de 26 protéines différentiellement présentes à la surface bactérienne et dont 2 seulement, InlJ et BnaG, présentent un motif LPXTG. En l'état, ces résultats ouvrent des pistes pour la sélection de cibles à étudier plus spécifiquement. Ainsi, parmi les protéines identifiées, celles portant un motif LPXTG représentent des cibles privilégiées. Ceci requerra l'obtention de mutants d'inactivation pour les 2 gènes codant pour InlJ et BnaG. L'analyse phénotypique de ces mutants permettra d'approfondir notre étude et de déterminer si la perte de l'inhibition observée avec le mutant BL23 *srtA2* est due directement à l'absence d'activité SrtA2 ou à un effet indirect lié à l'absence de ses 2 substrats, InlJ et ou BnaG, à la surface bactérienne.

- **Impact de *L. casei* BL23 sur la modulation de la réponse immunitaire innée de l'hôte**

La modulation de la réponse immunitaire est l'un des mécanismes utilisés par les bactéries probiotiques pour exercer leurs effets bénéfiques sur l'hôte (Amdekar et al., 2011 ; Lebeer et al., 2010 ; Tsai et al., 2012). La protection de la glande mammaire implique la production de certaines cytokines comme IL-6, IL-8, le facteur TNF-α, ainsi que l'expression de certains peptides antimicrobiens, en particulier TAP et LAP (Rainard et Riollet, 2006 ; Oviedo-Boyso et al., 2007).

L'objectif de cette phase du projet a été de déterminer si *L. casei* BL23 a la capacité de moduler la réponse immunitaire à *S. aureus* dans un modèle cellulaire CEMb en utilisant la lignée PS (choisie car elle répond mieux aux stimuli que la lignée MAC-T).

Ces résultats sont encore préliminaires et nécessitent des expérimentations supplémentaires. Pour l'heure, la co-incubation de *L. casei* BL23 et *S. aureus* N305 sur les CEMb PS a montré des résultats prometteurs en ce qui concerne la réduction de IL-6, IL-8, TNF-α, IL-1α et IL-1β, importants marqueurs de la réponse inflammatoire. La condition de post-incubation avec *L. casei* BL23 a également été testée afin d'évaluer la capacité de BL23 à traiter les infections causées par *S. aureus*. Cependant, aucune capacité significative à moduler la réponse immunitaire par *L. casei* BL23 n'a été observée dans cette condition.

Les résultats obtenus jusqu'ici constituent des données préliminaires permettant d'établir les conditions les plus appropriées pour l'évaluation de la contribution de *srtA2* de *L. casei* BL23 à la modulation de la réponse immunitaire. En outre, l'expression des gènes de cytokines a d'abord été évaluée par PCR quantitative et il est prévu d'effectuer la mesure des cytokines par ELISA pour confirmer les résultats. D'autres cytokines que celles testées ici pourront également être recherchées (par exemple, IL-10 ou IL-32).

Un autre fait important à considérer est que le système immunitaire de l'hôte répond de manière différente en fonction du type d'agent pathogène auquel il est exposé. En particulier, les infections mammaires provoquées par *S. aureus* et *E. coli* présentent des profils différents. L'infection causée par *E. coli* a un profil aigu, et peut être éliminée en quelques jours. A l'inverse, l'infection à *S. aureus* provoque une réponse inflammatoire généralement plus faible mais conduit fréquemment à une infection chronique, peut être en partie en raison de cette réaction inflammatoire plus modérée (Bannerman et al., 2004). Par conséquent, il est important de noter le type de réponse immunitaire dans notre modèle stimulé par d'autres pathogènes majeurs tels que *E. coli*.

Enfin, les probiotiques sont plus souvent envisagés dans une logique de prévention que de traitement. Nous allons évaluer l'impact du pré-traitement par *L. casei* BL23 sur la réponse inflammatoire des CEMb lors de l'infection par *S. aureus*.

➢ Conclusion finale

Les résultats obtenus dans cette thèse sont une première étape pour comprendre les mécanismes utilisés par *L. casei* BL23 pour inhiber l'internalisation de *S. aureus* dans les cellules épithéliales mammaires bovines. Tous ces résultats obtenus *in vitro* ne représentent évidemment pas le comportement global des probiotiques *in vivo*. Mais ces approches constituent un bon outil capable de cribler et d'identifier les meilleures souches pour la formulation d'un probiotique mammaire. La possibilité de développer un traitement alternatif

aux antibiotiques, utilisant les bactéries probiotiques de l'écosystème mammaire bovin, peut représenter une alternative efficace, sûre et peu coûteuse, pour réduire les pertes associées à la mammite, éviter l’émergence de pathogènes antibiorésistants et assurer le bien-être des animaux.

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ANEXOS

Table 1. Protein abundance ratios at the cell surface between the srtA2 mutant and *L. casei* BL23 wt determined by Peak Counting.

Protein ID	Protein name	Gene name	Ratio srtA2/WT
B3WEF7	Dihydroorotase	pyrC	3.31
B3WE86	ATPase component of ABC transporter with duplicated ATPase domains	LCABL_16060	2.50
B3WEF5	Carbamoyl-phosphate synthase large chain	carB	2.36
B3WEQ3	Putative uncharacterized protein	LCABL_17740	2.33
B3WEF8	Aspartate carbamoyltransferase	pyrB	2.30
B3WEF6	Carbamoyl-phosphate synthase small chain	pyrAA	2.13
B3WEF2	Orotate phosphoribosyltransferase	pyrE	2.00
B3WBL4	EIIC	manM	2.00
B3W9S6	CRISPR-associated endonuclease Cas9	cas9	2.00
B3W8E6	ABC-type multidrug transport system, ATPase and permease component	LCABL_06860	1.75
B3WCH6	Major tail protein, phi13 family	LCABL_09910	1.67
B3WEW6	Serine/threonine protein kinase (Putative	pkn1	1.43
B3WER8	Transcription elongation protein NusA (Transcription termination-antitermination factor NusA)	nusA	1.41
B3WAN2	tRNA-dihydrouridine synthase	LCABL_27000	1.40
B3W6Z0	Oxidoreductase	ylbE	1.36
B3WBW2	Phosphotransferase system sugar-specific EII component	PTS-EII	1.31
B3W6Z3	Putative uncharacterized protein	LCABL_07030	1.29
B3WDA7	Similar to cold shock protein, CspA family	csp3	1.27
B3WCM6	Ribosomal subunit interface protein	yfiA	1.26
B3WAM9	ATP-dependent Clp protease, ATP-binding subunit ClpC	clpC	1.25
B3WES0	DNA polymerase III PolC-type	polC	1.25
B3WEU8	Signal recognition particle-docking protein FtsY	ftsY	1.23
B3W6Y0	Beta-glucosides PTS, EIIABC	pts4ABC	1.23
B3WAI4	Putative uncharacterized protein	LCABL_26370	1.22
B3WBX0	Galactose-6-phosphate isomerase subunit lacA	lacA	1.17
B3W9Y3	Oleoyl-[acyl-carrier protein] thioesterase (Putative)	fat	1.14

B3W9W4	DNA mismatch repair protein MutS	mutS	1.14
B3WE50	Tagatose-6-phosphate kinase	fruK	1.13
B3WCP7	Thioredoxin reductase	trxB2	1.13
B3WBN3	Glutamine ABC transporter, ATP-binding protein	glnQ1	1.07
B3WEY4	Bifunctional protein FolD	folD	1.00
B3WAK7	50S ribosomal protein L14	rplN	0.92
B3W9A0	Glycogen phosphorylase	glgP	0.71
B3WF43	50S ribosomal protein L20	rplT	0.70
B3WF19	Phenylalanine--tRNA ligase alpha subunit	pheS	0.63
B3WE98	Glyceraldehyde 3-phosphate dehydrogenase	gapB	0.63
B3WEA8	ATP-dependent protease subunit HslV	hslV	0.63
B3W8X9	Putative citrate lyase alpha subunit (EC 4.1.3.6)	citF	0.60
B3W7V3	Pyruvate oxidase	cidC	0.57
B3WF02	Rhodanese-related sulfurtransferase	LCABL_18740	0.57
B3W8N5	Putative uncharacterized protein	LCABL_02770	0.56
B3WEI8	Aspartate aminotransferase	aspB	0.55
B3WBJ7	Argininosuccinate synthase	argG	0.54
B3WF93	Bifunctional purine biosynthesis protein PurH	purH	0.48
B3W8S9	Putative uncharacterized protein	LCABL_20210	0.44
B3WEG0	Bifunctional protein PyrR	pyrR1	0.43
B3WF92	Phosphoribosylamine--glycine ligase	purD	0.38
B3WF95	Phosphoribosylformylglycinamidine cyclo-ligase	purM	0.35
B3WAK8	30S ribosomal protein S17	rpsQ	0.33
B3WF98	Phosphoribosylformylglycinamidine synthase subunit PurQ	purQ	0.33
B3WCE2	Putative uncharacterized protein	LCABL_09570	0.33
B3WFA0	Phosphoribosylaminoimidazole-succinocarboxamide synthase	purC	0.29
B3WFA1	N5-carboxyaminoimidazole ribonucleotide synthase	purK1	0.26
B3WF96	Amidophosphoribosyltransferase	purF	0.18

B3WF94	Phosphoribosylglycinamide formyltransferase	purN	0.14

Table 2. Protein abundance ratios at the cell surface between the srtA2 mutant and *L. casei* BL23 wt determined by Spectral Counting.

Protein ID	Protein name	Gene name	Ratio srtA2/WT
B3WEF6	Carbamoyl-phosphate synthase small chain	pyrAA	-
B3WEF8	Aspartate carbamoyltransferase	pyrB	67.50
B3WEF2	Orotate phosphoribosyltransferase	pyrE	92.00
B3WEF7	Dihydroorotase	pyrC	97.50
B3W9Z1	Nucleoid-associated protein	LCABL_24440	18.00
B3WBU7	Oxidoreductase	ypjH	16.00
B3WEF5	Carbamoyl-phosphate synthase large chain	carB	33.62
B3WE82	TPR repeats containing protein	LCABL_16020	2.63
B3WBL4	EIIC	manM	3.17
B3W911	ABC transporter oligopeptide-binding protein (Oligopeptide/dipeptide ABC transporter oligopeptide/dipeptide-binding protein)	oppA	2.79
B3WE50	Tagatose-6-phosphate kinase	fruK	3.58
B3WE51	Transcription regulator of fructose operon	fruR	-
B3WAM9	ATP-dependent Clp protease ATP-binding subunit ClpC	clpC	1.89
B3WEV6	Oligopeptide ABC transporter ATP-binding protein	oppD	3.62
B3WES0	DNA polymerase III PolC-type	polC	4.57
B3W9W4	DNA mismatch repair protein MutS	mutS	2.96
B3WF19	Phenylalanine--tRNA ligase alpha subunit	pheS	0.06
B3W7V3	Pyruvate oxidase	cidC	0.00
B3W6R0	DNA gyrase subunit B	gyrB	1.66
B3W7F9	Replicative DNA helicase C	dnaC	8.00
B3WCP0	Phosphate-specific transport system accessory protein PhoU	phoU	0.07
B3W762	Aminopeptidase P	pP	-
B3WAG2	Phosphonates import ATP-binding protein PhnC	phnC	0.06
B3W744	Phosphotransferase system sugar-specific EIIB component	PTS-EIIB	0.00
B3WCE1	Putative uncharacterized protein	LCABL_09560	0.00

B3W880	FrdC protein	frdC	-
B3W8H6	Chromosome partitioning protein DNA-binding protein	parB2	-
B3W8Y6	Putative uncharacterized protein	LCABL_20880	0.00
B3WCC4	Aromatic amino acid specific aminotransferase	araT1	0.00
B3W7T4	67 kDa myosin-cross-reactive antigen-like protein	LCABL_04890	0.00
B3WF96	Amidophosphoribosyltransferase	purF	0.00
B3WE86	ATPase component of ABC transporter with duplicated ATPase domains	LCABL_16060	-
B3WF98	Phosphoribosylformylglycinamidine synthase subunit PurQ	purQ	0.00
B3W9G3	MocE	mocE	11.00
B3WDK0	ABC transporter ATP binding protein	ycfI	11.00
B3W828	Fructose/mannose phosphotransferase system IID component	fosD	0.00
B3WF94	Phosphoribosylglycinamide formyltransferase	purN	0.00
B3WD10	H+-K+-exchanging ATPase	pacL	0.00
B3WEF3	Orotidine 5'-phosphate decarboxylase	pyrF	-
B3WEF4	Dihydroorotate dehydrogenase	pyrD	-
B3WEG0	Bifunctional protein PyrR	pyrR1	0.09
B3WB22	Galactitol PTS EIIC	pts36C	3.00
B3W817	Cation-transporting ATPase E1-E2 family	pacL	3.41
B3WAP0	Hypoxanthine-guanine phosphoribosyltransferase	hpt	7.00
B3WD50	Predicted membrane protein	LCABL_12160	4.50
B3W9Z4	Putative uncharacterized protein	LCABL_24470	2.17
B3WF32	Ribosomal silencing factor RsfS	yqeL	2.32
B3W7G1	Adenylosuccinate synthetase	purA	0.05
B3WF06	Bifuntional dimerisation/transpeptidase penicillin-binding protein 2B	pbp2B2	3.75
B3WCB1	Low temperature requirement C protein also similar to B. subtilis YutG protein	ltrC	0.12
B3WDY8	YlmH (RNA-binding protein)	ylmH	2.07
B3WDP6	YueI protein	yueI	1.74
B3WAG1	Phosphonate ABC transporter substrate binding protein	phnD	0.13

B3WDZ9	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase 2	pfs	1.94
B3WF83	Beta-lactamase-like	LCABL_19560	0.11
B3WBI8	Putative uncharacterized protein	LCABL_30070	2.11
B3W8X9	Putative citrate lyase alpha subunit (EC 4.1.3.6)	citF	0.27
B3W998	Putative thiamine biosynthesis lipoprotein	apbE	1.80
B3W9W3	DNA mismatch repair protein MutL	mutL	-
B3W7M5	Alkaline shock protein	asp2	0.14
B3W9V8	Putative uncharacterized protein	LCABL_24110	-
B3WDN9	Putative uncharacterized protein	LCABL_14090	0.00
B3W912	SA2F01-3 protein	SA2F01-3	-
B3WFC7	Cellobiose PTS EIIA	pts20A	-
B3WC89	Putative uncharacterized protein	LCABL_08670	1.87
B3WE64	ATP-dependent 6-phosphofructokinase	pfkA	0.45
B3WEK7	Glycine--tRNA ligase alpha subunit	glyQ	0.00
B3WDP4	Chromosome segregation helicase (Putative)	cshA3	-
B3WF02	Rhodanese-related sulfurtransferase	LCABL_18740	0.21
B3WEH4	Transcriptional regulator LysR family (Transcriptional regulator HypR)	hypR	0.11
B3WFA2	N5-carboxyaminoimidazole ribonucleotide mutase	purE	0.07
B3WBK4	Cellobiose PTS EIIA	pts20A	3.50
B3WAV2	Proline iminopeptidase	pI	0.38
B3WCN1	Cell division protein FtsX	ftsX	0.21
B3WD20	Probable transcriptional regulatory protein LCABL	LCABL_11860	1.74
B3WF81	Adapter protein MecA	mecA	9.00
B3WBQ4	Gamma hydroxybutyrate dehydrogenase-like protein (Os02g0562700 protein) (Putative uncharacterized protein)	Os02g0562700	10.00
B3W7H3	YkpA protein	ykpA	6.33
B3WCB3	Peptidyl-prolyl cis-trans isomerase	ppiB	2.09
B3WEV5	Oligopeptide ABC transporter (ATP-binding protein)	oppF	0.44
B3W8D9	ABC-type multidrug transport system ATPase component	CcmA5	9.00

B3WD37	Putative uncharacterized protein	LCABL_12030	2.10
B3W757	Phosphotransferase system sugar-specific EII component	PTS-EII	1.77
B3WF95	Phosphoribosylformylglycinamidine cyclo-ligase	purM	0.04
B3W9I8	Acetyl-CoA carboxylase carboxyl transferase subunit alpha	accA	0.17
B3WC88	Putative uncharacterized protein ygaI	ygaI	1.81
B3WF92	Phosphoribosylamine--glycine ligase	purD	0.06
B3W9B1	Wzb	wzb	0.32
B3WB74	Putative dihydroorotase	dho	0.42
B3WBZ2	ABC-type uncharacterized transport system periplasmic component	LCABL_07680	4.13
B3WAE7	NAD-dependent epimerase/dehydratase:3-beta hydroxysteroid dehydrogenase/isomerase:NmrA-like	LCABL_26000	2.36
B3WFA1	N5-carboxyaminoimidazole ribonucleotide synthase	purK1	0.05
B3W8H8	Chromosome partitioning protein DNA-binding exonuclease	parB2	1.76
B3W8N5	Putative uncharacterized protein	LCABL_02770	0.23
B3WE57	Chaperone ClpB	clpB	1.72
B3WCK9	GMP reductase	guaC	0.46
B3WAT6	Putative ABC transporter ATP-binding protein	ybbL	-
B3WDW6	Cell shape-determining protein MreC	mreC	0.00
B3WDL9	ATP synthase epsilon chain	atpC	0.31
B3WF99	Conserved purine biosynthesis cluster protein	purS	0.00
B3WBT2	Lipase	lipA	-
B3W9K7	Putative uncharacterized protein	LCABL_23090	-
B3WCC1	Glycosyltransferase	rfaG	-
B3WC02	Putative uncharacterized protein	LCABL_07780	-
B3W7M0	Putative uncharacterized protein	LCABL_01870	-
B3W7B1	Putative uncharacterized protein	LCABL_01250	0.00
B3W9L2	ADP-ribose pyrophosphatase	LCABL_23140	0.00
B3WA30	Predicted dinucleotide-binding enzyme	LCABL_24830	-
B3W9B2	Wzr	wzr	-

B3WA31	Oxidoreductase (Possible Coenzyme F420-dependent N5N10-methylene tetrahydromethanopterin reductase and related flavin-dependent oxidoreductase)	yhbW	-
B3WCW2	N-acetylmuramoyl-L-alanine amidase	lys	0.00
B3WAN2	tRNA-dihydrouridine synthase	LCABL_27000	2.14
B3WAX8	Glycine betaine/carnitine/choline ABC transporter ATP-binding protein	choQ	11.00
B3WF93	Bifunctional purine biosynthesis protein PurH	purH	0.12
B3WFA0	Phosphoribosylaminoimidazole-succinocarboxamide synthase	purC	0.07
B3WAM4	30S ribosomal protein S12	rpsL	0.60
B3WEA1	Ribosome biogenesis GTPase A	ylqL	0.38
B3WF97	Phosphoribosylformylglycinamidine synthase subunit PurL	purL	0.07
B3WBW6	Putative uncharacterized protein	LCABL_07420	0.32
B3WER8	Transcription elongation protein NusA (Transcription termination-antitermination factor NusA)	nusA	1.54
B3WCM0	Putative competence-damage inducible protein	cinA	0.17
B3WAK1	30S ribosomal protein S5	rpsE	1.55
B3WE07	GCN5-related N-acetyltransferase	LCABL_15270	4.75
B3W907	ATP binding protein	oppD	2.20
B3W9A0	Glycogen phosphorylase	glgP	0.25
B3WAS5	HD superfamily phosphohydrolase	LCABL_27430	0.41
B3WF36	Predicted GTPase	LCABL_19080	0.27
B3WEY5	N utilization substance protein B homolog	nusB	0.45
B3WCM9	Peptide chain release factor 2	prfB	3.50
B3W9N3	Uncharacterized protein yuaG	yuaG	2.57
B3W9I9	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	accD	0.13
B3WF69	Putative uncharacterized protein	LCABL_19410	1.69
B3WAI4	Putative uncharacterized protein	LCABL_26370	1.47
B3WF86	ABC transporter related	LCABL_19590	4.33
B3W6Z3	Putative uncharacterized protein	LCABL_07030	1.91
B3WDK7	Peptide chain release factor 1	prfA	1.46
B3W6Y5	Cold shock protein A	cspA	0.38

B3WCR2	ATP-dependent Clp protease proteolytic subunit	clpP	1.58
B3WEB4	Formate C-acetyltransferase (Pyruvate formate-lyase) (Formate acetyltransferase)	pflB	1.41
B3WBW8	Tagatose 16-diphosphate aldolase	lacD2	1.60
B3W9U7	Putative uncharacterized protein	LCABL_23990	0.25
B3WE19	Dihydrolipoyl dehydrogenase	pdhD	0.42
B3W8P2	ABC-type sugar transport system periplasmic component	LCABL_02840	0.17
B3WEK5	DNA primase	dnaG	0.17

Table 3. Protein abundance ratios at the cell surface between the srtA2 mutant and *L. casei* BL23 wt determined by Extracted Ion Chromatogram (XIC).

Protein ID	Protein name	Gene name	Ratio srtA2/WT
B3WF06	Bifuntional dimerisation/transpeptidase penicillin-binding protein 2B	pbp2B2	6.27
B3W9M8	Putative uncharacterized protein yxbA	yxbA	5.20
B3WEF2	Orotate phosphoribosyltransferase	pyrE	4.45
B3WE00	Cysteine desulfurase	csd2	4.39
B3W978	dTDP-glucose 4,6-dehydratase	rmlB	3.83
B3WEF8	Aspartate carbamoyltransferase	pyrB	3.71
B3W7H3	YkpA protein	ykpA	3.56
B3WEF7	Dihydroorotase	pyrC	3.39
B3WE51	Transcription regulator of fructose operon	fruR	3.22
B3WEF6	Carbamoyl-phosphate synthase small chain	pyrAA	3.15
B3WDP1	Putative regulator	plcR	2.94
B3WEV0	Ribonuclease 3	rnc	2.90
B3WB22	Galactitol PTS, EIIC	pts36C	2.88
B3W8K0	Response regulator	rrp11	2.85
B3WAP0	Hypoxanthine-guanine phosphoribosyltransferase	hpt	2.73
B3WCP7	Thioredoxin reductase	trxB2	2.65
B3WEY0	Hemolysin A	tlyA	2.62
B3WDV7	Septation ring formation regulator ezrA	ezrA	2.60
B3W8V7	Serine--tRNA ligase	serS	2.60
B3WE82	TPR repeats containing protein	LCABL_16020	2.52
B3WBI8	Putative uncharacterized protein	LCABL_30070	2.49
B3WFA9	Putative uncharacterized protein	LCABL_19820	2.46
B3W7K1	Probable phosphoketolase	xpk	2.43
B3W6N4	Chromosomal replication initiator protein DnaA	dnaA	2.36
B3WEX8	DNA repair protein RecN	recN	2.35

B3WA51	Internalin-J	inlJ	2.35
B3WBI0	Ribosomal RNA large subunit methyltransferase H	rlmH	2.34
B3W868	L-sorbose-1-phosphate reductase	sorE	2.30
B3WA33	TRNA/rRNA methyltransferase	spoU	2.28
B3W971	GTPase HflX	ybnA	2.27
B3WBW2	Phosphotransferase system sugar-specific EII component	PTS-EII	2.26
B3WEQ5	Elongation factor 4	lepA	2.24
B3WBL4	EIIC	manM	2.22
B3WCR2	ATP-dependent Clp protease proteolytic subunit	clpP	2.22
B3WDQ6	Oligopeptide ABC transporter, substrate-binding lipoprotein	oppA	2.20
B3WEV1	Oligopeptide ABC trasporter substrate binding protein	oppA	2.19
B3W9Z4	Putative uncharacterized protein	LCABL_24470	2.17
B3WF38	YkcA (Glyoxalase/Bleomycin resistance protein/dioxygenase domain superfamily protein)	ykcA	2.15
B3WF32	Ribosomal silencing factor RsfS	yqeL	2.15
B3WBI6	Cadmium-/zinc-/cobalt-transporting ATPase	cadA	2.11
B3WC71	UPF0473 protein LCABL_08490	LCABL_08490	2.07
B3W8H1	Putative uncharacterized protein	LCABL_06650	2.07
B3W911	ABC transporter, oligopeptide-binding protein (Oligopeptide/dipeptide ABC transporter, oligopeptide/dipeptide-binding protein)	oppA	2.06
B3WCC6	YfnI	yfnI	2.05
B3WC98	Biotin-[acetyl-CoA-carboxylase] ligase	birA	2.00
B3W817	Cation-transporting ATPase, E1-E2 family	pacL	1.93
B3W8T4	Nicotinate phosphoribosyltransferase	nadC	1.91
B3WEH9	Predicted N6-adenine-specific DNA methylase	LCABL_16990	1.89
B3W992	Guanylate kinase	gmk2	1.86
B3WC40	Putative uncharacterized protein	LCABL_08180	1.86
B3WA35	Cysteine--tRNA ligase	cysS	1.83
B3WF18	Phenylalanine--tRNA ligase beta subunit	pheT	1.82
B3W7K6	Putative uncharacterized protein	LCABL_01730	1.79

B3WEW6	Serine/threonine protein kinase (Putative)	pkn1	1.76
B3WEU4	30S ribosomal protein S16	rpsP	1.75
B3WF10	YneR (Conserved protein YneR)	yneR	1.74
B3WA10	ATPase, E1-E2 type:Magnesium-translocating P-type ATPase	LCABL_24630	1.67
B3WDP9	L-serine dehydratase, iron-sulfur-dependent, alpha subunit	sdhA-2	1.62
B3W9U6	Putative uncharacterized protein aa23	aa23	1.61
B3WBJ1	Putative uncharacterized protein yycI	yycI	1.60
B3WAN2	tRNA-dihydrouridine synthase	LCABL_27000	1.59
B3WC74	Endonuclease MutS2	mutS2	1.59
B3W9S6	CRISPR-associated endonuclease Cas9	cas9	1.56
B3WAE2	Aldehyde dehydrogenase A	aldA	1.55
B3WD20	Probable transcriptional regulatory protein LCABL_11860	LCABL_11860	1.54
B3WE49	Fructose-specific phosphotransferase system, enzyme IIABC	fruA	1.52
B3WE10	UPF0356 protein LCABL_15300	LCABL_15300	1.51
B3WAL8	30S ribosomal protein S10	rpsJ	1.50
B3WA16	Ribonucleoside-triphosphate reductase	rtpR	1.49
B3WCE6	Transcriptional regulator, Cro/CI family	LCABL_09610	1.49
B3W8K4	UDP-N-acetylmuramyl-tripeptide synthetase	murE	1.47
B3W9N3	Uncharacterized protein yuaG	yuaG	1.47
B3W753	Beta-galactosidase 3	bgaC	1.47
B3WAV9	Putative uncharacterized protein	LCABL_27770	1.45
B3W7I3	6-phospho-beta-galactosidase	lacG	1.45
B3WCP4	HPr kinase/phosphorylase	hprK	1.44
B3WBY6	Putative uncharacterized protein	LCABL_07620	1.43
B3WC30	Thiol peroxidase (Hydroperoxide reductase, Peroxiredoxin)	tpx	1.36
B3WAK4	30S ribosomal protein S8	rpsH	1.35
B3W792	RbsB (Ribose ABC transporter) (Ribose-binding protein)	rbsB	1.34
B3W9Y8	Thymidylate kinase	tmk	1.32

B3WE13	Peptide deformylase	def	1.29
B3W827	Mannose-6-phosphate isomerase	pmi	1.29
B3W793	Ribokinase	rbsK	1.27
B3WAK3	50S ribosomal protein L6	rplF	0.80
B3WF19	Phenylalanine--tRNA ligase alpha subunit	pheS	0.80
B3WFA6	Phosphocarrier protein HPr (Histidine-containing protein)	ptsH	0.79
B3WA82	Gamma-glutamyl phosphate reductase	proA	0.77
B3W8T0	Cation transporting P-type ATPase	pacL2	0.75
B3W6X9	Alpha, alpha-phosphotrehalase	treA	0.75
B3W8J7	Putative uncharacterized protein ybjB	ybjB	0.75
B3WAK7	50S ribosomal protein L14	rplN	0.74
B3W7L9	Phosphoglycerate dehydrogenase	serA2	0.74
B3W8J8	Inosine-5'-monophosphate dehydrogenase	guaB	0.73
B3WDP6	YueI protein	yueI	0.73
B3WC66	ATP-dependent RNA helicase	srmB	0.72
B3W6U9	Glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	opuA	0.72
B3WCJ6	UPF0210 protein LCABL_10110	LCABL_10110	0.72
B3W7F8	50S ribosomal protein L9	rplI	0.70
B3WEQ8	Protein GrpE	grpE	0.70
B3WC58	Queuine tRNA-ribosyltransferase	tgt	0.70
B3W9A3	Glucose-1-phosphate adenylyltransferase	glgC	0.70
B3WBP4	Galactoside O-acetyltransferase	thgA3	0.70
B3W979	RmlC	rmlC	0.70
B3WAV1	Methionine--tRNA ligase	metS	0.69
B3WDM7	Succinyl-diaminopimelate desuccinylase (Lmo0265 protein)	dapE	0.68
B3W6S8	Surface antigen	LCABL_00230	0.68
B3WDJ5	Dipeptidase D-type (U34 family)	pepD2	0.68
B3W9D2	Wze	wze	0.68

B3W9P7	Acetate kinase	ackA	0.67
B3WA09	50S ribosomal protein L11	rplK	0.67
B3W8B9	Phospotransferase system PTS, mannose-specific IIB component	levB	0.66
B3W6R4	30S ribosomal protein S6	rpsF	0.66
B3WEA1	Ribosome biogenesis GTPase A	ylqL	0.66
B3WE81	Histone-like DNA-binding protein HU	hbsU	0.66
B3WEY6	Alkaline shock protein	asp3	0.65
B3W8N8	NADH oxidase	nox	0.64
B3WAL3	30S ribosomal protein S19	rpsS	0.64
B3WEI8	Aspartate aminotransferase	aspB	0.64
B3W6Z1	ARA1 protein	ara1	0.64
B3W7V2	Fructose-bisphosphate aldolase	fba	0.64
B3W9A4	1,4-alpha-glucan branching enzyme	glgB	0.64
B3WB73	L-seryl-tRNA(Sec) selenium transferase (Putative)	selA	0.63
B3WA61	Cysteine aminopeptidase C2 (Bleomycin hydrolase)	pepC2	0.62
B3W9J5	Malonyl CoA-acyl carrier protein transacylase	fabD	0.61
B3WBW3	Phosphotransferase system, galactitol-specific IIB component	LCABL_07390	0.61
B3WER5	Translation initiation factor IF-2	infB	0.60
B3WEY7	Elongation factor P	efp	0.59
B3W739	NADH peroxidase	npr	0.58
B3WBJ7	Argininosuccinate synthase	argG	0.58
B3WE19	Dihydrolipoyl dehydrogenase	pdhD	0.57
B3W7T7	PTS-dependent dihydroxyacetone kinase, phosphotransferase subunit dhaM (Phosphotransferase enzyme IIA component) (PTS system EIIA component)	dhaM	0.56
B3WF39	Lactoylglutathione lyase related lyase	LCABL_19110	0.55
B3WF41	NADPH:quinone reductase related Zn-dependent oxidoreductase	LCABL_19130	0.54
B3WE46	Glutamine ABC transporter, ATP-binding protein	glnQ3	0.54
B3WAV5	L-2-hydroxyisocaproate dehydrogenase	hicD3	0.54
B3W9J9	Transcriptional regulator, marR family	marR	0.53

B3W7J7	Putative uncharacterized protein yhgE	yhgE	0.53
B3WBG0	Exopolyphosphatase-related protein	LCABL_29790	0.53
B3W7C3	D-lactate dehydrogenase	ldhD	0.53
B3WAJ3	30S ribosomal protein S11	rpsK	0.52
B3WAJ1	50S ribosomal protein L17	rplQ	0.52
B3W9K2	Butanol dehydrogenase	bdh	0.52
B3WA07	Succinate-semialdehyde dehydrogenase (NAD(P)+)	gabD	0.51
B3WB74	Putative dihydroorotase	dho	0.51
B3WCX9	Beta-phospho-glucomutase	pgmA	0.50
B3WCB3	Peptidyl-prolyl cis-trans isomerase	ppiB	0.50
B3W8P4	Beta-N-acetylglucosaminidase	glcNAcase	0.50
B3WF40	Putative uncharacterized protein yahD	yahD	0.50
B3W983	UDP-glucose 4-epimerase	galE3	0.49
B3WAV2	Proline iminopeptidase	pepI	0.49
B3WF27	6-phosphogluconate dehydrogenase, decarboxylating	gndA	0.49
B3W9X6	PTS system, IIB component	celA	0.49
B3WEZ1	50S ribosomal protein L21	rplU	0.49
B3WCY2	Maltose ABC transporter substrate binding protein	malE	0.48
B3WCJ1	Glutathione peroxidase	bsaA	0.47
B3W8U8	Phosphoenolpyruvate carboxykinase (ATP)	pck	0.46
B3WE85	Putative uncharacterized protein	LCABL_16050	0.46
B3W9D3	Wzd	wzd	0.46
B3WDZ9	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase 2	pfs	0.45
B3W756	Phosphotransferase system sugar-specific EIID component	PTS-EIID	0.45
B3WCW4	Glyceraldehyde 3-phosphate dehydrogenase	gap-1	0.44
B3WA25	NADH dehydrogenase	ndh2	0.44
B3WBX9	Putative uncharacterized protein yneB	yneB	0.44
B3W847	PTS, EIIA	frvA	0.43

B3WEH3	Ribonucleoside-diphosphate reductase, beta chain	nrdF	0.41
B3W8K5	Aspartate racemase	racD	0.40
B3W8X9	Putative citrate lyase alpha subunit (EC 4.1.3.6)	citF	0.40
B3WE64	ATP-dependent 6-phosphofructokinase	pfkA	0.39
B3W7I5	Galactokinase	galK	0.39
B3WDY9	Cell-division initiation protein (Septum placement)	divIVA	0.39
B3WC53	S-ribosylhomocysteine lyase	luxS	0.39
B3WF92	Phosphoribosylamine--glycine ligase	purD	0.39
B3WAM4	30S ribosomal protein S12	rpsL	0.38
B3WCB1	Low temperature requirement C protein, also similar to B. subtilis YutG protein	ltrC	0.36
B3WA36	Lactate dehydrogenase (Oxidoreductase)	loxD	0.34
B3WAP8	L-lactate dehydrogenase	ldh	0.34
B3WCN9	Phosphate import ATP-binding protein PstB	pstB2	0.34
B3W8F4	Predicted flavoprotein	LCABL_06480	0.29
B3W8K7	IolR	iolR	0.28
B3WEB3	DNA topoisomerase 4 subunit A	parC	0.28
B3W9K0	(3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase ((3R)-hydroxymyristoyl ACP dehydrase)	fabZ	0.27
B3WD72	Putative uncharacterized protein	LCABL_12380	0.27
B3WER7	Transcription elongation factor NusA	nusA	0.26
B3WBL3	EIID	manN	0.24
B3W956	Dipeptidase	pepD3	0.24
B3W7G1	Adenylosuccinate synthetase	purA	0.21
B3WF97	Phosphoribosylformylglycinamidine synthase subunit PurL	purL	0.21
B3WCS4	Conserved domain protein	LCABL_10900	0.19
B3WAG1	Phosphonate ABC transporter, substrate binding protein	phnD	0.18
B3WF95	Phosphoribosylformylglycinamidine cyclo-ligase	purM	0.13
B3WF70	Foldase protein PrsA	prtM	0.13
B3WF93	Bifunctional purine biosynthesis protein PurH	purH	0.13

B3W8N5	Putative uncharacterized protein	LCABL_02770	0.10
B3W8Y1	Citrate lyase acyl carrier protein	citD	0.04