UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Estudo comparativo de proteínas de superfície de *Mycoplasma hyopneumoniae* e *Mycoplasma flocculare*

Tese de Doutorado

Lais Del Prá Netto Machado

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS como requisito parcial para a obtenção do título de Doutora em Ciências

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Orientador: Prof. Dr. Henrique Bunselmeyer Ferreira

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"*Je n'ai pas d'espoir de sortir par moi-même de ma solitude. La pierre n'a pas d'espoir d'être autre chose que pierre, mais en collaborant, elle s'assemble et devient Temple"*

Antoine de Saint-Exupéry

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LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

%	porcentagem
~	aproximadamente
β	beta
µm	micrometros
ºC	grau(s) Celsius
2DE-MS/MS	eletroforese em gel bidimensional e espectrometria de massas em tandem, do inglês, *two-dimensional gel electrophoresis and tandem-mass spectrometry*
ATCC	Coleção de Culturas-Tipo Norte Americana, do inglês, *American Type Culture Collection*
C	Citosina
CDRS	complexo das doenças respiratórias de suínos
CDSs	sequências de DNA codificadoras, do inglês *coding DNA sequences*
DNA	ácido desoxirribonucleico, do inglês, *deoxyribonucleic acid*
ELISA	ensaio de imunoabsorção ligado à enzima, do inglês, *enzyme-linked immunosorbent assay*
G	guanina
G + C	guanina mais citosina
Ig	Imunoglobulinas
IgG	imunoglobulina G
IL	Interleucina
Kb	quilobase(s)
LC-MS/MS	cromatografia líquida acoplada a espectrometria de massas em tandem, do em inglês *liquid chromatography-tandem mass spectrometry*
MIB	proteína ligadora de Ig de micoplasma, do inglês, *Mycoplasma Ig binding protein*
MIP	protease de Ig de micoplasma, do inglês, *Mycoplasma Ig protease*
METs	armadilhas extracelulares de macrófagos, do inglês, *macrophage extracellular traps*
mRNA	ácido ribonucleico mensageiro, do inglês, *messenger ribonucleic acid*
NETs	armadilhas extracelulares de neutrófilos, do inglês, *neutrophil extracellular traps*
OC	Agrupamentos de fases abertas de leitura, do inglês, *open reading frame clusters*

ORF	fase aberta de leitura, do inglês, *open Reading frame*
pb	par(es) de bases
PCR	reação em cadeia da polimerase, do inglês, *polymerase chain reaction*
PEP	*Porcine enzootic pneumonia*
PES	pneumonia enzoótica suína
pH	potencial de hidrogênio
PRRSV	vírus da síndrome suína reprodutiva e respiratória, do inglês, *porcine reproductive and respiratory syndrome virus*
RNA	ácido ribonucleico ribossômico, do inglês, *ribosomal ribonucleic acid*
SCD	sequências codificadoras de DNA
TRS	trato respiratório de suínos

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RESUMO

Mycoplasma hyopneumoniae e *Mycoplasma flocculare* são bactérias geneticamente semelhantes que coabitam o trato respiratório de suínos (TRS). Estes micoplasmas compartilham a maioria dos genes que codificam fatores de virulência conhecidos ou preditos. Contudo, *M. hyopneumoniae* é o principal agente etiológico da pneumonia enzoótica suína (PES), enquanto nenhuma doença foi, até o momento, associada à presença de *M. flocculare* no TRS. Além disto, algumas linhagens de *M. hyopneumoniae* também podem diferir em patogenicidade e virulência. Até o momento, estudos comparativos genômicos e transcritômicos realizados com linhagens de *M. hyopneumoniae*, e *M. flocculare* não explicam totalmente as diferenças na patogênese ou virulência entre essas linhagens e espécies. A fim de identificar potenciais determinantes da PES e fornecer uma melhor compreensão das interações patógeno-hospedeiro, o proteoma total de duas linhagens de *M. hyopneumoniae*, uma patogênica (7448) e outra não patogênica (J), e *M. flocculare* foram comparadas. Uma abordagem com frações celulares combinada com espectrometria de massas (LC-MS/MS) foi utilizada a fim de analisar comparativamente os proteomas das frações enriquecidas de proteínas citoplasmáticas e de superfície. A detecção média foi de aproximadamente 50% dos proteomas preditos para *M. hyopneumoniae* e *M. flocculare*. Muitas das proteínas identificadas foram diferencialmente representadas em *M. hyopneumoniae* 7448 em comparação com *M. hyopneumoniae* J e *M. flocculare*, incluindo potenciais determinantes da PES, como adesinas, proteases e transportadores de membrana. Algumas destas adesinas podem sofrer processamentos proteolíticos pós-traducionais na superfície de *M. hyopneumoniae* e *M. flocculare*, produzindo diferentes proteoformas com diferentes propriedades de adesão. Baseado nos dados de LC-MS/MS, foi avaliado o processamento proteolítico diferencial entre os ortológos de adesinas mais abundantes (p97 e p216) ou proteínas de superfície relacionadas a adesão (DnaK, p46 e transportador ABC) das linhagens de *M. hyopneumoniae* (7448 e J) e *M. flocculare*. Foram observados eventos de clivagem diferencial entre as linhagens de *M. hyopneumoniae* e *M. flocculare*, e entre a superfície e citoplasma. Os dados obtidos evidenciam um cenário complexo de múltiplas proteoformas antigênicas de proteínas relacionadas com a adesão, que são diferenciais entre as linhagens de *M. hyopneumoniae* e *M. flocculare*, alterando a arquitetura da superfície e provavelmente contribuindo com a virulência e patogenicidade observada.

ABSTRACT

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* are genetically similar bacteria, which coinhabit the porcine respiratory tract. These mycoplasmas share most of the genes known or predicted virulence factors. However, *M. hyopneumoniae* is considered the primary pathogen of porcine enzootic pneumonia (PEP), while no disease was associated with the presence of *M. flocculare*. Furthermore, some *M. hyopneumoniae* strains could also differ in pathogenicity and virulence. To date, comparative genomic and transcriptomic studies performed with *M. hyopneumoniae* strains and *M. flocculare* do not fully explain the differences the differences in pathogenesis and virulence among the strains and species. To identify potential PEP determinants and provide novel insights on mycoplasma-host interactions, the whole cell proteomes of two *M. hyopneumoniae* strains, one pathogenic (7448) and other non-pathogenic (J), and *M. flocculare* were compared. A cell fractioning approach combined with mass spectrometry (LC-MS/MS) proteomics was used to comparative analyze cytoplasmic and surface-enriched protein fractions. Average detection of ~50% of the predicted proteomes of *M. hyopneumoniae* 7448 and J, and *M. flocculare* were achieved. Many of the identified proteins were differentially represented in *M. hyopneumoniae* 7448 in comparison to *M. hyopneumoniae* J and *M. flocculare*, including potential PEP determinants, such as adhesins, proteases, and redox-balancing proteins, among others. Some of these adhesins undergo post-translational endoproteolytic processing on the surface of *M. hyopneumoniae* and *M. flocculare*, producing differential proteoforms with differential adhesion properties. Based on LC-MS/MS data, we assessed differential proteolytic processing among orthologs of the five most abundant adhesins (p97 and p216) or adhesion-related surface proteins (DnaK, p46, and ABC transporter xylose-binding lipoprotein) from *M. hyopneumoniae* strains 7448 (pathogenic) and J (non-pathogenic), and *M. flocculare*. It was demonstrated that not only *bona fide* adhesins, but also adhesion-related proteins undergo proteolytical processing, both in the cytoplasm and surface. Overall, our data provided evidence of a complex scenario of multiple antigenic proteoforms of adhesion-related proteins, that are differential among *M. hyopneumoniae* strains and *M. flocculare*, altering the surface architecture and likely contributing to virulence and pathogenicity.

1. INTRODUÇÃO

1.1 A classe Mollicutes

Os organismos da classe Mollicutes fazem parte de um grupo de microrganismos procarióticos que são distintos da maioria das bactérias por possuírem características diferenciadas como a ausência de parede celular, tamanho e genoma reduzidos. Diversos estudos levaram à melhor compreensão da estrutura da membrana celular, do genoma e das rotas metabólicas destes organismos, e ao reconhecimento deles como os menores e mais simples organismos autorreplicativos (RAZIN & HAYFLICK, 2010). Nas subseções a seguir (1.1.1 a 1.1.3), será abordada esta classe de bactérias, com ênfase em espécies do trato respiratório de suínos (TRS), como *Mycoplasma hyopneumoniae* e *Mycoplasma flocculare*, que são as espécies objeto deste trabalho.

1.1.1 Taxonomia, evolução, morfologia e fisiologia

Micoplasmas ou molicutes são termos utilizados para referir-se a todas as espécies que compõe a classe Mollicutes (RAZIN, 2006). Tais microrganismos não apresentam parede celular de peptideoglicanos e são deficientes em algumas vias metabólicas, características que acompanharam a redução do tamanho de seus genomas ao longo da evolução (CITTI & BLANCHARD, 2013; VEDYAYKIN *et al*., 2019). Com base em estudos do tamanho e estrutura do genoma (MOROWITZ & WALLACE, 1973), foi sugerido que os micoplasmas são organismos descendentes de uma bactéria ancestral prévia ao surgimento da parede celular de peptidoglicano (GLASS *et al*., 2017; RAZIN & HAYFLICK, 2010). Todavia, estudos filogenéticos e filogenômicos de micoplasmas demonstraram a evolução de micoplasmas a partir de uma bactéria Gram-positiva ancestral, envolvendo a redução do genoma e perda da parede celular (CITTI & BLANCHARD, 2013; GUPTA & OREN, 2020; IPOUTCHA *et al*., 2019; MINION, 2002; SIRAND-PUGNET *et al*., 2007).

Quando comparados com outras bactérias de maiores genomas, os micoplasmas aparentam ser organismos mais simples, com os genomas variando entre 600 e 2200 kb. De forma geral, espécies dos gêneros *Acholeplasma*, *Anaeroplasma*, *Asteroleplasma* e *Spiroplasma* possuem os genomas maiores (~2200 kb), enquanto as dos gêneros *Mycoplasma*, *Ureaplasma*, *Mesoplasma* e *Phytoplasma* possuem os genomas mais reduzidos. Reconstruções evolutivas mostram que os micoplasmas formam um clado monofilético dentro do filo Tenericutes, onde se dividem em dois grandes ramos: o ramo

AAP, formado por espécies dos gêneros *Acholeplasma*, *Anaeroplasma*, *Asteroleplasma* e do grupo *Phytoplasma*; e o ramo SEM que incluí as espécies dos gêneros *Spiroplasma*, *Mesoplasma*, *Ureaplasma* e *Mycoplasma* (CITTI *et al.*, 2018; SIRAND-PUGNET *et al.*, 2007).

Morfologicamente micoplasmas são caracterizados pela ausência de parede celular e são delimitados por uma única membrana plasmática de formato predominantemente esférico. Entretanto, outros formatos podem ser observados como forma de pera, estruturas com forma de balão com estruturas de ponta terminal, filamentos de vários comprimentos e filamentos helicoidais. Para manutenção da morfologia na ausência de parede celular de peptideoglicanos, os micoplasmas contam com a presença de um citoesqueleto que auxilia a manutenção do formato celular e participação na divisão celular (RAZIN & HAYFLICK, 2010; VEDYAYKIN *et al*., 2019). Além disto, o citoesqueleto, juntamente com proteínas de adesão, pode estar envolvido na motilidade de alguns micoplasmas, como *Mycoplasma mobile* e *Mycoplasma gallisepticum*. Outra característica de micoplasmas é capacidade de ultrapassar membranas utilizadas para a filtração, devido ao seu tamanho reduzido, enquanto outras bactérias são retidas (VEDYAYKIN *et al.*, 2019).

Micoplasmas são organismos fastidiosos e de multiplicação lenta em condições laboratoriais, que acarreta na dificuldade de cultivo e identificação destas bactérias em amostras clínicas (CITTI & BLANCHARD, 2013; MAY & BROWN, 2018). O sequenciamento e a análise de genomas demonstraram que alguns micoplasmas são deficientes em genes envolvidos na síntese de aminoácidos, necessitando o fornecimento exógeno pela utilização de meios enriquecidos (por exemplo, utilização de infusão de coração-cérebro, peptona, extrato de levedura) e suplementados com uma variedade de soros (ex. soro fetal bovino, soro suíno) para superar tais deficiências (ADLER & BERG, 1960; BARBER & FABRICANT, 1962; COOK *et al.*, 2016; EDWARD, 1971; JANSSON, 1971; KOBISCH & FRIIS, 1996; TAMURA; KURAMASU; TAJIMA, 1975). Diversos esforços foram realizados para definir os requisitos nutricionais de micoplasmas, utilizando primeiramente métodos de cultivo clássico que foram substituídos por estudos genômicos que permitiram a definição de genes e sistemas metabólicos específicos disponíveis por estas bactérias. Além da deficiência de síntese de aminoácidos, os micoplasmas possuem uma dependência de suplemento exógeno de ácidos graxos e colesterol, substratos importantes para a síntese da membrana celular e possuem um processo respiratório truncado, ausente

do ciclo completo de ácido tricarboxílico, quinonas e citocromos (RAZIN & HAYFLICK, 2010; VEDYAYKIN *et al.*, 2019).

1.1.2 Ecologia

Os micoplasmas são amplamente distribuídos na natureza, encontrados em seres humanos, mamíferos, répteis, peixes, artrópodes e plantas, podem ser consideradas parte da microbiota ou causar infecções (CITTI *et al*., 2018; KANDAVELMANI & PIRAMANAYAGAM, 2019; LANAO; CHAKRABORTY; PEARSON-SHAVER, 2021; RAZIN & HAYFLICK, 2010). Os habitats primários de micoplasmas em seres humanos e animais são superfícies do trato respiratório e urogenitais, olhos, canal digestivo, glândulas mamárias e articulações (LANAO; CHAKRABORTY; PEARSON-SHAVER, 2021; RAZIN & HAYFLICK, 2010). Sabe-se que espécies de micoplasmas são restritas a hospedeiros e tecidos específicos, o que explica a falta de modelos animais que poderiam mimetizar infecções em condições laboratoriais (CITTI & BLANCHARD, 2013; RAZIN & HAYFLICK, 2010). Apesar disto, já foram reportados casos da ocorrência de doenças causadas por micoplasmas em diferentes tecidos ao preferencial e em hospedeiros não usuais. Por exemplo, *M. pneumoniae* responsável por infecções do trato respiratório em humanos e que já foi associado a outros sítios como pele, sistema nervoso central, sangue, coração, trato urogenital e articulações (LANAO; CHAKRABORTY; PEARSON-SHAVER, 2021; VEDYAYKIN *et al.*, 2019) e *Mycoplasma canis*, uma espécie canina que pode fazer parte da microbiota de bovinos, quando há contato direto entre estes animais (PITCHER & NICHOLAS, 2005).

Os micoplasmas são parasitas intracelulares não obrigatórios que se aderem à superfície de células e podendo posteriormente penetrar nas células do hospedeiro, como, como o *Mycoplasma penetrans* (KANDAVELMANI & PIRAMANAYAGAM, 2019). Em estudo *in vitro* realizado recentemente por Raymond e colaboradores (2018) demonstrou também a capacidade de *M. hyopneumoniae*, um patógeno historicamente considerado extracelular, de penetrar em células epiteliais suínas. O nicho intracelular protege o patógeno do sistema imune e da ação de muitos antibióticos, mecanismo de patogenicidade que será discutido em maiores detalhes na subseção a seguir (1.1.3. Patogenicidade e virulência).

1.1.3 Patogenicidade e virulência

Até meados do século XX, o conceito de patógeno era definido como a capacidade de um microrganismo causar infecção no hospedeiro (BYNDLOSS & BÄUMLER, 2018; JOHNSON, 2018). Os fatores de virulência, incluindo macromoléculas como as exotoxinas, endotoxinas e cápsulas; eram considerados os responsáveis por causar os sintomas da doença e, portanto, acreditava-se estarem presentes apenas nas bactérias consideradas patogênicas. Entretanto, diversas doenças infecciosas não são causadas apenas pela ação dos organismos ditos como patógenos primários e seus fatores de virulência (EZEPCHUK, 2017; JOHNSON, 2018). Sabe-se que os sintomas causados por infecções bacterianas estão relacionados com a resposta do sistema imune do hospedeiro frente ao patógeno. Nos últimos quarenta anos, os conceitos de virulência e patogenicidade foram sendo atualizados, sendo a virulência definida como o resultado da interação entre o patógeno e o hospedeiro infectado e a capacidade do microrganismo de modular as respostas imune inata e adaptativa do hospedeiro, além dos danos causados ao tecido do hospedeiro. Desta forma, a virulência é relacionada à capacidade do patógeno de causar dano tecidual ao hospedeiro (por exemplo, aquela proporcionada por exotoxinas), e moléculas e estruturas que permitem que o patógeno escape do sistema imune do hospedeiro (por exemplo, cápsulas, biofilmes) (DIARD & HARDT, 2017; EZEPCHUK, 2017; JOHNSON, 2018). Fatores expressos por microrganismos que são responsáveis pela interação patógeno-hospedeiro e auxiliam no desenvolvimento da infecção são denominados fatores de virulência. Os fatores de virulência estão associados à função de: (*i*) adesão, mecanismos ou complexos que possuem um papel fundamental em se ligar as células do hospedeiro, como as adesinas; (*ii*) invasão e sobrevivência dentro das células do hospedeiro, como os patógenos intracelulares, que permitem a evasão dos sistema imune e resistência a antimicrobianos; (*iii*) evasão do sistema imune, pela produção de biofilmes e cápsulas, que podem impedir a ação de fagocitose e lise; (*iv*) modulação do sistema imune do hospedeiro, gerando uma hipo- ou hiper-resposta imune do hospedeiro (EZEPCHUK, 2017; JOHNSON, 2018; MARTÍNEZ *et al.*, 2019; RIBET & COSSART, 2015).

Micoplasmas exibem uma maior propensão de desenvolver mutações, devido à ausência da atividade da exonuclease de revisão 3’ para 5’ e a menor quantidade de genes relacionados ao reparo do ácido desoxirribonucleico (DNA) (RAZIN; YOGEV; NAOT, 1998), em comparação a outras bactérias, o que pode garantir que desenvolvam resistência a determinados antibióticos mais rapidamente (KANDAVELMANI &

PIRAMANAYAGAM, 2019; RAZIN; YOGEV; NAOT, 1998; WAITES; LYSNYANSKY; BÉBÉAR, 2014). A ausência de parede celular faz com que, micoplasmas sejam naturalmente resistentes a antibióticos que atuam na síntese da parede celular como β-lactâmicos, glicopeptídeos e fosfomicina (GAUTIER-BOUCHARDON, 2018). Além disto, são naturalmente resistentes à rifamicina, pela mutação natural no gene *rpoB* da subunidade β da RNA-polimerase, que inibe a ligação do antibiótico ao sítio de ação (CHERNOV *et al.*, 2018; GAURIVAUD; LAIGRET; BOVE, 1996; GOLDSTEIN, 2014). Assim, as tetraciclinas, macrolídeos, fluoroquinolonas e pleuromutilinas são os antimicrobianos amplamente utilizados no tratamento de animais e seres humanos em infecções causadas por micoplasmas (BOULIANNE *et al.*, 2020; MAES *et al.*, 2020; MCVEY; KENNEDY; CHENGAPPA, 2013; SULYOK *et al.*, 2017). Entretanto, resistência ou redução na sensibilidade a antimicrobianos pode ser observada em estudos *in vitro* e *in vivo* (GAUTIER-BOUCHARDON *et al.*, 2002; LE CARROU *et al.*, 2006a, 2006b; SULYOK *et al.*, 2017; WU *et al.*, 2005; ZHANG, N. *et al.*, 2017).

A adesão de micoplasmas às células do hospedeiro é considerada pré-requisito para a colonização e desenvolvimento da infecção. Por tais motivos, diversos estudos buscam a identificação e caracterização das proteínas encontradas na superfície destas bactérias (RAZIN & HAYFLICK, 2010). Algumas espécies de micoplasmas, como *M. pneumoniae*, *Mycoplasma fermentans*, *M. genitalium* e *M. penetrans* contam com o auxílio de uma organela periférica especializada que possui uma função de ancoramento (chamada no inglês de *tip attachment structure*). Além da função de adesão, esta organela também está relacionada com a motilidade destes micoplasmas (HE *et al.*, 2016; ROACHFORD *et al.*, 2019). As adesinas são proteínas relacionadas à adesão de micoplasmas às células do hospedeiro. Diversos estudos vêm buscando caracterizar e avaliar o papel de adesinas em relação a patogênese e virulência (CHRISTODOULIDES *et al.*, 2018; HE *et al.*, 2016; LIU, W. *et al.*, 2019; RAYMOND *et al.*, 2015; ROACHFORD *et al.*, 2019; TACCHI *et al.*, 2016; WIDJAJA *et al.*, 2017, 2020; XIONG *et al.*, 2016b, 2016a; YU *et al.*, 2018a, 2018b). *M. pneumoniae* é uma das espécies mais estudadas quanto ao repertório e funções de adesinas e outras proteínas relacionadas com a adesão ou o ancoramento (BALISH, 2006; CHAUDHRY; VARSHNEY; MALHOTRA, 2007; JIANG *et al.*, 2021; NAKANE *et al.*, 2021; VIZARRAGA *et al.*, 2020). Sabe-se que o processo de adesão não está diretamente relacionado a uma proteína exclusiva e sim à ação de um conjunto de proteínas. Em *M.*

pneumoniae a proteína P1 possui um papel fundamental na adesão e conjuntamente com as proteínas P30 (fatores de adesão A, B e C), P40, P90, P65 e os polipeptídeos HMW 1-5, formam um complexo responsável pela adesão (HE *et al.*, 2016). De forma semelhante, o *M. genitalium* possui uma série de proteínas relacionadas com a adesão e mutantes dessa espécie demonstraram uma redução na motilidade e citoaderência (ROACHFORD *et al.*, 2019).

Micoplasmas podem induzir respostas pró-inflamatórias pela secreção de toxinas, antígenos de superfície e outros mecanismos não muito claros (CHRISTODOULIDES *et al.*, 2018). Estudos relacionados com a variação antigênica e caracterização molecular de proteínas presentes na superfície de micoplasmas têm ganhado bastante foco para a melhor compreensão da patogenicidade destas bactérias (HEGDE *et al.*, 2018; KANDAVELMANI & PIRAMANAYAGAM, 2019; ROACHFORD *et al.*, 2019; ROACHFORD; NELSON; MOHAPATRA, 2017; XIONG *et al.*, 2016b, 2016a; ZHANG, J. *et al.*, 2017). As lipoproteínas são um grupo de proteínas que se destaca em relação à variação antigênica em micoplasmas, e geralmente exibem um domínio lipídico e um peptídico (CHRISTODOULIDES *et al.*, 2018). O domínio lipídico normalmente está associado à membrana plasmática, e o domínio peptídico está relacionado à variação antigênica (ROACHFORD *et al.*, 2019). Esta variação antigênica está associada a alterações no tamanho de lipoproteínas, resultado do número de até 60 repetições em tandem de sequências correspondentes a 10 a 19 aminoácidos nos genes que codificam lipoproteínas (CHRISTODOULIDES *et al.*, 2018).

As famílias de lipoproteínas variáveis podem ser observadas em diversas espécies de micoplasmas, como *Mycoplasma hyorhinis*, uma espécie patógena em suínos (XIONG *et al.*, 2016b, 2016a). Diversos antígenos de superfície de lipoproteínas geram uma forte resposta imunológica e tem papel chave entre a bactéria e o sistema imune do hospedeiro (XIONG *et al.*, 2016b, 2016a). As lipoproteínas de micoplasmas geram um processo pró-inflamatório e ativação do sistema imune tanto em células epiteliais como em células imunes. A liberação de citocinas pró-inflamatórias, como a IL-1β, IL-6, IL-8 e IL-2, induzidas pelas lipoproteínas podem levar ao recrutamento de células imunes, como neutrófilos. O mecanismo de evasão do sistema imune por estas proteínas incluem a indução de apoptose de células monocíticas e linfocíticas, formando uma camada protetora contra a ação de anticorpos que inibem o crescimento, e contra a fagocitose pelos macrófagos. Apesar

de incitar potente resposta inflamatória, as lipoproteínas também podem induzir a liberação de citocinas anti-inflamatórias, como a IL-10, auxiliando na evasão do sistema imune e prolongando a infecção (CHRISTODOULIDES *et al.*, 2018).

Além da evasão proporcionada pelos mecanismos de variação antigênica, micoplasmas podem evadir o sistema imune do hospedeiro pela captura, proporcionada pelo sistema MIB (proteína ligadora de Ig de micoplasmas, do inglês, *Mycoplasma Ig binding protein*) e/ou clivagem de anticorpos, sistema MIP (protease de Ig de micoplasmas, do inglês, *Mycoplasma Ig protease*) que levam à diminuição da fagocitose pelo sistema complemento (ARFI *et al.*, 2016). A clivagem de imunoglobulinas acontece pela ação de proteases, uma classe de proteínas também consideradas fatores de virulência. Está estratégia foi descrita para diversas espécies de micoplasmas, como *Mycoplasma synoviae*, *M. genitalium*, *M. pneumoniae*, *M. gallisepticum*, *Mycoplasma mycoides* e *Ureaplasma urealyticum* (ARFI *et al.*, 2016; BLÖTZ *et al.*, 2020; GROVER *et al.*, 2015; NARAT *et al.*, 2011; SPOONER; RUSSELL; THIRKELL, 1992).

Além disso, o envolvimento de proteases na diversificação do repertório de proteínas da superfície já foi descrito ou considerado em algumas espécies de micoplasmas patógenos de seres humanos (*M. pneumoniae* e *M. fermentans*) (DAVIS & WISE, 2002; WIDJAJA *et al.*, 2020), animais (*M. hyopneumoniae*, *Mycoplasma capricolum* e *M. mycoide*s) (GANTER *et al.*, 2019; TACCHI *et al.*, 2016; ZHAO *et al.*, 2012) e plantas (*Spiroplasma citri*) (DUBRANA *et al.*, 2017). Essa proteólise está relacionada com a modificação de antígenos na superfície de micoplasmas, como estratégia de variação antigênica, clivando adesinas, lipoproteínas, e/ou proteínas que exercem uma função canônica no citoplasma e que podem exercer uma função diferente na superfície bacterianas (CITTI; NOUVEL; BARANOWSKI, 2010; DUBRANA *et al.*, 2017; GANTER *et al.*, 2019; GAURIVAUD *et al.*, 2018; JAROCKI *et al.*, 2019a; LI *et al.*, 2019). O processamento proteolítico (clivagem) de proteínas é uma modificação pós-traducional que gera diferentes fragmentos proteicos, chamados de proteoformas, que podem apresentar diferentes propriedades de adesão dos micoplasmas às células do hospedeiro e a componentes da matriz extracelular, como plasminogênio, heparina e actina. (BERRY *et al.*, 2017; CHANG *et al.*, 2011; DUBRANA *et al.*, 2017; RAYMOND *et al.*, 2015; TACCHI *et al.*, 2014, 2016; WIDJAJA *et al.*, 2015, 2017, 2020).

Proteases de *M. hyopneumoniae* (LI *et al.*, 2019; PAES *et al.*, 2017a), *M. hyorhinis* (PADDENBERG *et al.*, 1998), *M. penetrans* (BENDJENNAT *et al.*, 1997, 1999), *M. gallisepticum* (XU *et al.*, 2015) e *M. pneumoniae* (SOMARAJAN; KANNAN; BASEMAN, 2010) também podem ter efeito citotóxico e levar apoptose de células do hospedeiro via diferentes mecanismos (LI *et al.*, 2019; MAY & BROWN, 2018; PAES *et al.*, 2017a). As proteases e peptidases podem ser secretadas (PAES *et al.*, 2017b; ROACHFORD *et al.*, 2019), possuindo um papel importante na modulação do sistema imune (ARFI *et al.*, 2016; STAATS *et al.*, 2007), obtenção de nutrientes (STAATS *et al.*, 2007) e o processamento proteolítico de proteínas na superfície de micoplasmas e do hospedeiro (GANTER *et al.*, 2019; JAROCKI *et al.*, 2019b).

Um potencial fator de virulência de bactérias é a degradação de DNA e RNA do hospedeiro por nucleases (KOONIN; MAKAROVA; WOLF, 2017). Nesse aspecto, experimentos *in vitro* utilizando nucleases recombinantes de *M. gallisepticum*, *M. hyopneumoniae* e *Mycoplasma bovis* incubadas com células eucarióticas demonstraram diminuição da viabilidade e indução da apoptose destas células, sugerindo o papel citotóxico deste grupo de proteínas (LI *et al.*, 2018, 2019; XU *et al.*, 2015; ZHANG *et al.*, 2016). Além disso, essas nucleases podem estar associadas à membrana de micoplasmas. Assim, além de causar danos às células do hospedeiro, essa ação pode disponibilizar pequenos oligonucleotídeos e bases livres para os micoplasmas, que são incapazes de sintetizar tanto bases púricas como pirimídicas devido às limitações nas respectivas vias biossintéticas (BIZARRO & SCHUCK, 2007; QIN; CHEN; YOU, 2019; WANG *et al.*, 2001).

As nucleases secretadas ou associadas à membrana de *Mycoplasma hominis* (CACCIOTTO *et al.*, 2019), *M. bovis* (GONDAIRA *et al.*, 2017; MITIKU *et al.*, 2018; ZHANG *et al.*, 2016), *M. hyopneumoniae* (LI *et al.*, 2019), *M. pneumoniae* (YAMAMOTO *et al.*, 2017) também podem degradar armadilhas extracelulares de neutrófilos (NETs, do inglês, *neutrophil extracellular traps*), e macrófagos (METs, do inglês, *macrophage extracellular traps*) em *M. hyopneumoniae* (HENTHORN; MINION; SAHIN, 2018), que são mecanismos da imunidade inata do hospedeiro para conter a infecção bacteriana. As NETs e METs são estruturas extracelulares compostas por cromatina condensada e proteínas granulares com a função de impedir a disseminação das bactérias, criando uma armadilha, e concentrar agentes antimicrobianos para degradar fatores de virulência e matar a bactéria (PAPAYANNOPOULOS, 2018). Como o maior componente das NETs e METs é o DNA,

as nucleases desses micoplasmas também podem influenciar na virulência, evadindo o sistema imune e rapidamente degradar estas estruturas (CACCIOTTO *et al.*, 2019; GONDAIRA *et al.*, 2017; HENTHORN; MINION; SAHIN, 2018; LI *et al.*, 2019; MITIKU *et al.*, 2018; YAMAMOTO *et al.*, 2017; ZHANG *et al.*, 2016).

Algumas espécies de micoplasmas também podem ser encontradas intracelularmente. A invasão das células do hospedeiro permite a proteção destes micoplasmas à ação do sistema imune do hospedeiro, e à atividade de antimicrobianos, possibilitando a permanência no citoplasma da célula do hospedeiro por mais tempo (ANDREEV *et al.*, 1995; DUŠANIĆ *et al.*, 2009; KIM; LEE; KO, 2019; MERWE; PRYSLIAK; PEREZ-CASAL, 2010; NUNOYA *et al.*, 2020; RAYMOND *et al.*, 2018a). Micoplasmas intracelulares podem interagir com componentes do citoplasma, como o citoesqueleto, levando a desestruturação da célula hospedeira (NUNOYA *et al.*, 2020; RAYMOND *et al.*, 2018a) e de transportadores de membrana, facilitando a disseminação célula a célula do patógeno e consequentemente a progressão da infecção (KIM; LEE; KO, 2019). Além disto, alterações em proteínas de processos relacionados ao metabolismo, tradução e resposta ao estresse oxidativo, foram observadas em *M. gallisepticum* durante a interiorização e foram associadas à adaptação, citotoxicidade, e comprometimento da integridade e permeabilidade da membrana celular do hospedeiro, facilitando a entrada da bactéria (MATYUSHKINA *et al.*, 2016). Assim, a evasão do sistema imune de micoplasmas por diferentes estratégias, como a invasão as células do hospedeiro podem explicar a caraterística de cronicidade observada nas infecções causadas por estes organismos (KIM; LEE; KO, 2019; MATYUSHKINA *et al.*, 2016; NUNOYA *et al.*, 2020; RAYMOND *et al.*, 2018a, 2018b).

1.2 Micoplasmas do trato respiratório de suínos

A presença de bactérias no TRS pode indicar um quadro de infecção, principalmente quando associadas à sinais clínicos e lesões pulmonares características de pneumonia. Todavia, diversas espécies bacterianas podem compor a flora normal do TRS, visto que já foram identificadas em animais que não apresentassem lesões pulmonares e/ou sinais clínicos de pneumonia (HUANG *et al.*, 2019; SIQUEIRA *et al.*, 2017). Entretanto, observam-se variações da diversidade bacteriana quando comparados pulmões de suínos com e sem sinais clínicos de infecção (HUANG *et al.*, 2019; SIQUEIRA *et al.*, 2017), além

da possível associação de algumas espécies bacterianas com a saúde, redução dos riscos e desfecho da infecção nos suínos (HUANG *et al.*, 2019).

Estudos recentes (HUANG *et al.*, 2019; SIQUEIRA *et al.*, 2017), buscaram identificar comunidades bactérias presentes em pulmões de suínos sadios (sem lesões) e com lesões, utilizando amostras de lavados broncoalveolares. Siqueira e colaboradores (2017) observaram uma distribuição uniforme das famílias bacterianas isoladas dos pulmões de suínos com sinais sugestivos de PES, enquanto uma população bacteriana mais diversas foi identificada em pulmões de animais sadios. Tais resultados foram corroborados no estudo realizado por Huang e colaboradores (2019), que descreve uma diversidade bacteriana reduzida nas amostras em que foram observadas lesões pulmonares. Apesar de tais achados, micoplasmas foram identificados em todas as amostras analisadas (pulmões com e sem sinais de lesão), e é o gênero mais abundante em suínos com lesões pulmonares, demonstrando a importância desse gênero no TRS e prevalência na maioria as granjas produtoras de suínos ao redor do mundo.

Siqueira e colaboradores (2017) identificaram a família Mycoplasmataceae como a mais abundante, tanto nas amostras com como nas sem lesões pulmonares. Dentre as espécies identificadas *M. hyopneumoniae*, conhecidamente associada a doenças respiratórias de suínos, foi responsável por 95% e 47% das leituras nos grupos com lesões pulmonares e sem lesões, respectivamente. *M. flocculare*, outra importante espécie de micoplasmas não associadas, até o momento, a doenças respiratórias em suínos, também foi identificada nos lavados de ambos os grupos, todavia em menores quantidades (0,6% em suínos com lesões pulmonares e 2% em suínos sem lesões pulmonares).

1.2.1 *M. hyopneumoniae* e *M. flocculare*

M. hyopneumoniae e *M. flocculare* são espécies de micoplasmas isoladas do TRS. Enquanto *M. hyopneumoniae* é o agente causador da pneumonia enzoótica suína (PES), possuindo alta prevalência no mundo inteiro e causando grandes perdas econômicas, nenhuma doença foi associada à presença de *M. flocculare* até o momento (BETLACH *et al*., 2019; FERRARINI *et al*., 2016; RAZIN & HERRMANN, 2002; SIQUEIRA *et al*., 2013). *M. hyopneumoniae* e *M. flocculare* pertencem ao filo *Tenericutes*, classe *Mollicutes*, ordem *Mycoplasmatales* e a família *Mycoplasmataceae* (RAZIN & HERRMANN, 2002). Análises filogenéticas comparativas entre *M. hyopneumoniae* e *M. flocculare* com base na sequência completa de 16S rRNA (STEMKE *et al.*, 1992), e filogenômicas (SIQUEIRA *et*

al., 2013) indicaram uma relação próxima entre estas duas espécies. A importância de *M. flocculare* está associada a proximidade antigênica e relação filogenética com *M. hyopneumoniae* (BETLACH *et al*., 2019; FERRARINI *et al*., 2016; RAZIN & HERRMANN, 2002; SIQUEIRA *et al*., 2013).

M. hyopneumoniae foi primeiramente isolado por Goodwing e colaboradores, no Reino Unido (1965), e por MARE e SWITZER (1965), nos Estados Unidos. *M. hyopneumoniae* possui morfologia celular redonda ou oval, com um diâmetro médio de 0,2 µm. *M. hyopneumoniae*, *M. flocculare* e *M. hyorhinis* podem ser cultivados em meio artificial Friis. O cultivo de *M. hyopneumoniae* pode ser realizado em meio artificial Friis, entretanto o crescimento é considerado mais lento quando comparados a outros micoplasmas suínos, levando em torno de 4 a 15 dias (KOBISCH & FRIIS, 1996). O crescimento é observado pela acidificação e consequente alteração da cor do meio pela utilização de um indicador de pH (KOBISCH & FRIIS, 1996).

M. flocculare, outra espécie de micoplasmas que habita o TRS, porém nenhuma doença foi associada a presença desta bactéria. A presença nos pulmões aparentemente demonstra uma duração restrita, e *M. flocculare* tende a permanecer na cavidade nasal dos suínos, podendo ser encontrado ao longo da vida do animal. O crescimento também pode ser feito em meio de cultura Friis. *M. flocculare* pode crescer a temperaturas mais baixas (30ºC), uma característica provavelmente relacionada a afinidade pelo trato respiratório superior. A morfologia celular é apresentada por um polimorfismo e em meio de cultura sólido, as colônias apresentam tamanhos irregulares, assim como em *M. hyopneumoniae*.

1.2.2 Pneumonia enzoótica suína (PES)

Diversas bactérias podem ser identificadas no TRS com e sem sinais clínicos e/ou lesões pulmonares características de infecção (SIQUEIRA *et al.*, 2017). Dentre estas, podemos citar *M. hyopneumoniae* que é a espécie considerada o agente etiológico da PES, e principal agente do complexo das doenças respiratórias dos suínos (CDRS). Devido aos custos causados pela PES, esforços foram realizados para obtenção de vacinas, tratamento e métodos sensíveis de detecção do organismo em amostras clínicas que contribuíram a criação de protocolos de controle e erradicação da PES (HOLST; YESKE; PIETERS, 2015; MAES *et al.*, 2018).

A PES causada por *M. hyopneumoniae* normalmente não apresenta sinais clínicos agudos, e pode se manter assintomática por longos períodos (MAES *et al.*, 2018; TAKEUTI

et al., 2017). Os sinais clínicos causados pela infecção por *M. hyopneumoniae* são geralmente intermitentes, variam na sua intensidade, apresentam tosse seca e podem durar semanas e até meses, mas em casos mais simples a infecção pode se manter subclínica, sem apresentar sintomas respiratórios ou lesões pulmonares (MAES *et al.*, 2018). A tosse é uma consequência direta das lesões pulmonares causadas pela infecção por *M. hyopneumoniae*, que consiste em consolidação roxas a cinzas afetando principalmente os lobos médios e apicais. Manejo, condições ambientais e linhagem de *M. hyopneumoniae* são diferentes fatores que podem estar envolvidos na intensidade dos sintomas e severidade das lesões (CORREA VALENCIA, 2018; GARCIA-MORANTE *et al.*, 2015; PRODANOV-RADULOVIĆ *et al.*, 2020). Apesar do impacto da variabilidade de linhagens em relação à severidade das lesões pulmonares não ser completamente compreendido, foi evidenciado que a coinfecção causada por mais de uma linhagem resulta em lesões pulmonares mais severas (BETLACH *et al.*, 2019; MAES *et al.*, 2018; MICHIELS *et al.*, 2017b). As lesões macroscópicas observadas e os sintomas clínicos não são exclusivas da infecção causada por *M. hyopneumoniae*, e outros patógenos devem ser considerados para o diagnóstico diferencial (BARALDI *et al.*, 2019; LUEHRS *et al.*, 2017).

M. hyopneumoniae pode ser identificado na mucosa da traqueia, brônquios e bronquíolos (MAES *et al.*, 2018). Determinar a prevalência exata da PES causada por *M. hyopneumoniae* é difícil, devido aos métodos de diagnósticos serem considerados imprecisos, além da sensibilidade ser variada de acordo com a amostra utilizada, metodologia empregada e limitações da técnica (PIETERS; DANIELS; ROVIRA, 2017). A transmissão ocorre pelo contato direto entre suínos infectados e animais susceptíveis a infecção (MAES *et al.*, 2018). Apesar de serem escassos os dados epidemiológicos no Brasil, estudos recentes detectaram a presença de *M. hyopneumoniae* em 32,2% dos suínos com quadros clínicos de infecções respiratórias e em 50% dos animais provenientes de granjas que relataram surtos do CDRS (MORÉS *et al.*, 2015; SCHMIDT *et al.*, 2016).

Após a adesão de *M. hyopneumoniae* ao TRS, a indução à ciliostase, perda dos cílios e perda da função de células do epitélio (Figura 1) (BETLACH *et al.*, 2019; DEBEY & ROSS, 1994; KWON; CHOI; CHAE, 2002), propícia a infecção a agentes secundários, como vírus e outras bactérias (ZIMMERMAN *et al.*, 2019). Nestes casos os sinais clínicos observados podem ser mais severos, como dificuldade de respiração, febre, anorexia, letargia e até morte (MAES *et al.*, 2018). Os quadros clínicos que envolvem vários agentes

infecciosos (vírus e/ou bactérias), associados a fatores de risco como estresses ambientais, diferenças nos sistemas de produção e medidas preventivas são comumente referenciados de CDRS (CHAE, 2016). Os patógenos predominantemente associados ao CDRS são *M. hyopneumoniae*, *Pasteurella multocida* (MORÉS *et al.*, 2015; SCHMIDT *et al.*, 2016), o vírus da síndrome suína reprodutiva e respiratória (PRRSV), o circovírus suíno tipo 2, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *M. hyorhinis*, e *Streptococcus suis* (CHEONG *et al.*, 2017; HERNANDEZ-GARCIA *et al.*, 2017; JUNIOR *et al.*, 2015; LI *et al.*, 2016; SAVIĆ *et al.*, 2015).

(A) (B)

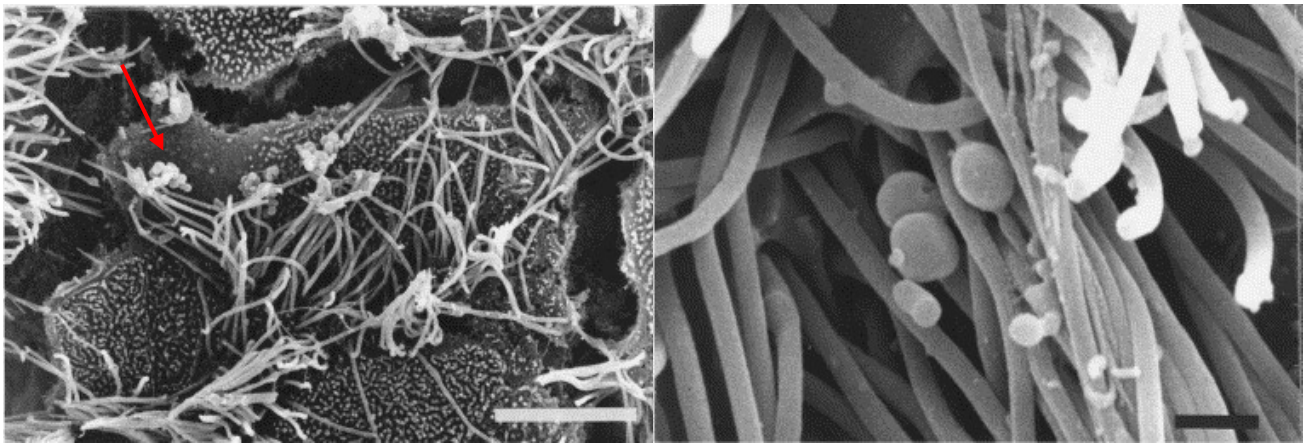

Figura 1. Eletromicrografia do cultivo de células ciliadas com *M. hyopneumoniae*. Microscopia eletrônica de varredura mostrando a aderência e danos às células ciliadas infectadas com a linhagem patogênica 91-3 de *M. hyopneumoniae*. (A) *M. hyopneumoniae* (indicado pela seta) aderido aos cílios induzindo o dano. Barra de escala = 5 µm. (B) cílios danificados e divididos longitudinalmente. Barra de escala = 0,5 µm. Figura adaptada de Young *et al*. (2000).

Os sinais clínicos não são exclusivos da PES e, portanto, é necessário que seja feito o diagnóstico laboratorial (CHAE, 2016). O isolamento do patógeno pelo cultivo dos tecidos de lesões pulmonares ainda continua sendo o método considerado padrão-ouro, entretanto, a necessidade de meios de cultura especializados, o alto custo associado a técnica, a contaminação frequente por outras bactérias e a baixa sensibilidade são alguns dos problemas desta abordagem (PIETERS & MAES, 2019). Apesar disto, estão sendo feitos esforços para controlar a contaminação por outras bactérias em meios de cultura para *M. hyopneumoniae*, como o desenvolvimento de meios de cultura seletivos, auxiliando no isolamento de linhagens que podem ser utilizadas na pesquisa, diagnóstico, desenvolvimento de vacinas, e avaliação dos testes de sensibilidade a antimicrobianos (COOK *et al.*, 2016).

Além do isolamento de *M. hyopneumoniae*, testes moleculares, como a reação em cadeia da polimerase (PCR), permitem a detecção de *M. hyopneumoniae* em diversas amostras com uma alta sensibilidade e especificidade (LIU, L. *et al.*, 2019; PIETERS; DANIELS; ROVIRA, 2017). Vários ensaios de PCR, como PCR convencional, PCR em tempo real e PCR tipo *nested* foram desenvolvidos e validados para a identificação de *M. hyopneumoniae*. A alta precisão e sensibilidade são vantagens da utilização desta técnica. Além disto, a coleta das amostras pode ser realizada tanto em animais vivos como abatidos (CALSAMIGLIA; PIJOAN; TRIGO, 1999; CARON; OUARDANI; DEA, 2000; DUBOSSON *et al.*, 2004; FOUROUR *et al.*, 2018; HARASAWA *et al.*, 1991; LIU, L. *et al.*, 2019; STÄRK; NICOLET; FREY, 1998; STRAIT *et al.*, 2008; TAKEUTI; DE BARCELLOS; PIETERS, 2017; VERDIN *et al.*, 2000). Entretanto, a sensibilidade do ensaio de PCR varia de acordo com a amostra analisada, amostras do trato respiratório inferior, principalmente àquelas em que lesões pulmonares características da infecção são observadas, apresentam maior sensibilidade do que amostras do trato respiratório superior, assim como *swabs* nasais e fluídos orais (PIETERS; DANIELS; ROVIRA, 2017; SPONHEIM *et al.*, 2020; TAKEUTI; DE BARCELLOS; PIETERS, 2017; VILALTA *et al.*, 2020). Os principais genes de escolha para a identificação de *M. hyopneumoniae* pelo ensaio de PCR estão relacionadas às proteínas de membrana com fatores de virulência bem caracterizados, como a P97, P46 e P102, o que aumentaria a probabilidade destes genes estarem presentes em todos os isolados patogênicos (MAROIS *et al.*, 2010; STRAIT *et al.*, 2008). *Kits* comerciais para a detecção de *M. hyopneumoniae* por PCR em tempo real também estão disponíveis, como o VetMAX™ *M. hyopneumoniae* produzido pela Applied Biosystems (Thermo Fisher Scientific, EUA) e genesig® Advanced Kit (Genesig, Reino Unido).

O diagnóstico da PES também pode ser realizado por testes sorológicos, comumente utilizados pela facilidade de obtenção de amostras de soro de suínos e facilidade na testagem (PIETERS & MAES, 2019). Vários testes sorológicos estão disponíveis para a identificação de IgG específicos, chamados de ELISA (ensaio de imunoabsorção enzimática, do inglês *Enzyme-Linked Immunosorbent Assay*) e variam de acordo com o antígeno utilizado (extratos celulares totais ou proteínas individuais) (DING; ZHOU; WANG, 2019; ERLANDSON *et al.*, 2005; LIU *et al.*, 2016; OKADA *et al.*, 2005; PIETERS & MAES, 2019; PROKEŠ *et al.*, 2012). Diversos *kits* ELISA comerciais estão disponíveis para a

detecção sorológica de *M. hyopneumoniae* e apesar de utilizarem diferentes antígenos, a sensibilidade é similar entre eles (PIETERS; DANIELS; ROVIRA, 2017). Entretanto, uma das desvantagens é o número relativamente elevado de resultados falso-negativos (de até 81%) (ERLANDSON *et al.*, 2005; PETERSEN *et al.*, 2016). Além disto, os anticorpos podem demorar de 3 a 8 semanas para serem detectados após a exposição experimental de suínos à *M. hyopneumoniae* e possuírem baixa sensibilidade no começo da infecção (PIETERS; DANIELS; ROVIRA, 2017). Os diagnósticos sorológicos podem ser difíceis de ser interpretados, pois à presença de anticorpos IgG detectados pelo teste de ELISA, podem ser provenientes da absorção de anticorpos maternos pelos leitões (BANDRICK *et al.*, 2008; WILSON *et al.*, 2013), ocorrência de reações cruzadas com outros micoplasmas que podem ser identificados no TRS, como *M. flocculare* e *M. hyorhinis* (PETERSEN *et al.*, 2016), ou gerados após a vacinação, e não apenas pela infecção (ERLANDSON *et al.*, 2005). Além disto, os níveis de anticorpos contra *M. hyopneumoniae* são reduzidos na fase crônica da doença e não são mais detectados (ERLANDSON *et al.*, 2005).

As medidas de controle e prevenção consistem em um conjunto de estratégias adotadas na suinocultura a fim de diminuir ou evitar a PES e outras doenças infecciosas. Evitar a introdução de *M. hyopneumoniae* é o primeiro passo para evitar a contaminação em granjas negativas e, em granjas acometidas, adotar medidas de erradicação e prevenção da disseminação é fundamental para diminuir os prejuízos econômicos causados por estas doenças. Diversas medidas podem ser adotadas para prevenir a disseminação de doenças infecciosas, como sistemas de filtração de ar, desinfecção e descontaminação de superfícies e fômites (CORREA VALENCIA, 2018; HOLST; YESKE; PIETERS, 2015; NATHUES *et al.*, 2016; ROBBINS *et al.*, 2019). Vários protocolos para a eliminação de *M. hyopneumoniae* das granjas já foram descritos, incluindo o despovoamento total e repovoamento, despovoamento parcial, fechamento das granjas e utilização de medicamentos antimicrobianos (HOLST; YESKE; PIETERS, 2015). Além disto, a testagem de animais provenientes de outras granjas é especialmente importante, pelo alto risco de introdução de *M. hyopneumoniae* ao rebanho (CORREA VALENCIA, 2018; HOLST; YESKE; PIETERS, 2015; NATHUES *et al.*, 2016; ROBBINS *et al.*, 2019).

Devido à resistência natural de micoplasmas a antibióticos β-lactâmicos, o uso de antimicrobianos potencialmente ativos a *M. hyopneumoniae* são recomendados, como macrolídeos, lincosamidas, tetraciclinas, pleuromutilinas, fluoroquinolonas e

aminoglicosídeos. A administração dos antimicrobianos pode ser feita através da alimentação, suplementada na ração ou na água dos suínos, e/ou por via parental. Apesar do uso de antimicrobianos adequados e melhora no quadro clínico, o tratamento apenas reduz a carga bacteriana, mas não a elimina totalmente (HOLST; YESKE; PIETERS, 2015; MAES *et al.*, 2020; PIETERS & MAES, 2019). O uso de antimicrobianos aumenta o risco de seleção de organismos resistentes e de espécies bacterianas da microbiota do animal, portanto deve-se ter cautela no tratamento antimicrobiano em infecções causadas por *M. hyopneumoniae* (GONZAGA *et al.*, 2020; HOLST; YESKE; PIETERS, 2015; LE CARROU *et al.*, 2006a, 2006b; MAES *et al.*, 2020; PIETERS & MAES, 2019).

A vacinação é outra medida preventiva importante para controlar as infecções causadas por *M. hyopneumoniae* (MORÉS *et al.*, 2015; SIMIONATTO *et al.*, 2013; TAKEUTI *et al.*, 2017). A maioria das vacinas comerciais consiste em células inteiras inativadas (bacterinas) com adjuvantes que são administradas por via intramuscular (TAO *et al.*, 2019). O efeito da vacinação por bacterinas varia de acordo com adjuvantes (VIRGINIO *et al.*, 2017) e a linhagem utilizada (MATTHIJS *et al.*, 2019a; VILLARREAL *et al.*, 2011). A tabela 1 demonstra as principais vacinas aprovadas para *M. hyopneumoniae* e as diferentes linhagens utilizadas.

Entretanto, o aumento de anticorpos no soro de suínos após a vacinação não garante a imunização protetiva, ou seja, não previnem a colonização de *M. hyopneumoniae*. As vantagens associadas à vacinação estão relacionadas à redução das perdas de performance dos suínos, redução da severidade dos sinais clínicos e lesões pulmonares (ARSENAKIS *et al.*, 2016; CVJETKOVIĆ *et al.*, 2018; FISCH *et al.*, 2016; MAES *et al.*, 2018; MATTHIJS *et al.*, 2019b; MICHIELS *et al.*, 2017a, 2017b; OH *et al.*, 2019; TAO *et al.*, 2019; VILLARREAL *et al.*, 2012; WILSON *et al.*, 2013). Outras desvantagens das vacinas de bacterinas são a ausência de informações completas dos antígenos expressos, a necessidade de meios de culturas enriquecidos que aumentam os custos de produção (DUIVON *et al.*, 2018; MARCHIORO *et al.*, 2012).

Tabela 1. Vacinas aprovadas para *M. hyopneumoniae*.

Nome comum	Linhagem de *M. hyopneumoniae*	Fabricante	Ano de aprovação
Vacina inativada	J	Boehringer Ingelheim Vetmedica, GmbH	2017
	J	Boehringer Ingelheim Vetmedica, Inc.	2017
	P	Protatek International, Inc.	2016
	J	Intervet International, B.V.	2016
	DJ-166	China Animal Husbandry Industry Co., Ltd.	2016
	BQ14	Merial, Inc.	2015
	J	Laborations HIPRA, S.A.	2015
	P-5722-3, I	Zoetis, Inc.	2014
	P-5722-3	Harbin Pharmaceutical Group Holding Co., Ltd.	2014
	P-5722-3, II	Zoetis, Inc.	2013
	J	Boehringer Ingelheim Vetmedica, GmbH	2013
	P	Protatek International, Inc.,	2012
	J	Boehringer Ingelheim Vetmedica GmbH	2012
	J	Intervet International, B.V.	2011
	BQ14	Merial, Inc.	2011
	J	Laborations HIPRA, S.A.	2010
	P-5722-3	Pfizer, Inc.	2010
Vacina inativada com adjuvante composto	P	Protatek International, Inc.	2017
	P	Protatek International, Inc.	2012
Vacina viva	RM48	EBVAC	2017
	168	Nanjing Tianbang Bio-industry Co., Ltd.	2016
	168	Fuzhou Da Bei Nong Biotech	2016
	168	Guizhou Fu Si Te Biotech	2016
	168	QYH Biotech Company, Ltd.	2015
	RM48	QiLu Animal Health Products Co., Ltd.	2015
	RM48	Shandong Lvdu Bio-technique Industry	2015

Tabela adaptada de Tao *et al.* (2019)

Devido às desvantagens das vacinas convencionais, vacinas polivalentes utilizando tecnologias de DNA recombinante poderiam fornecer melhores resultados. Inicialmente, a busca de vacinas recombinantes teve enfoque em apenas um antígeno do patógeno, pela simplicidade na composição e proteção efetiva. Para tanto, a identificação de fatores de virulência e imunogênicos são o ponto de partida para o desenvolvimento de vacinas de engenharia genética. Deste modo, os pesquisadores se concentraram em genes relacionados

a fatores de virulência, como a nuclease MnuA e a proteína de choque térmico 70 (HSP70) (VIRGINIO *et al.*, 2014) e proteínas de adesão ao cílio do epitélio respiratório de suínos, em especial as adesinas, por ser um importante fator de virulência além de fundamental para o desenvolvimento da doença (OVERESCH & KUHNERT, 2017; SIMIONATTO *et al*., 2013; VIRGINIO *et al*., 2014). Nos últimos anos, o foco principal dos estudos é em fatores de adesão, como as adesinas P97, P36, P42, P46 e P95 (DE OLIVEIRA *et al*., 2017; OVERESCH & KUHNERT, 2017). As vacinas de DNA tem sido uma estratégia em desenvolvimento, são mais seguras e podem ser administradas por diferentes vias, como intramuscular, oral, subcutânea ou intranasal (CHEN *et al.*, 2008). Diversos estudos demonstram uma forte resposta imunológica, desejável no desenvolvimento de vacinas, pela imunização com antígenos relacionados a fatores de virulência a proteína P42 (CHEN *et al.*, 2003; GALLI *et al.*, 2012), proteína de choque térmico HSP70 e nuclease MnuA (VIRGINIO *et al.*, 2014), P37 e P46 (GALLI *et al.*, 2012).

1.3 Genômica comparativa e funcional de *M. hyopneumoniae* e *M. flocculare*

O aumento do número de sequências bacterianas completamente sequenciadas de diferentes linhagens e espécies, e disponibilização destas sequencias de DNA em diversas bases de dados, proporcionaram avanços na compreensão da fisiologia, patogenicidade e virulência (RASKIN *et al.*, 2006). Até 31/05/2022, 159 genomas completos de espécies do gênero *Mycoplasma* estão depositados e disponíveis na base de dados GenBank (https://www.ncbi.nlm.nih.gov/datasets/genomes/?taxon=2093&utm_source=gquery&utm_medium=referral&utm_campaign=KnownItemSensor:taxname), e avanços na transcritômica, na proteômica e em análises metabólicas e epigenéticas estão acelerando uma caracterização detalhada para a melhor compreensão da patogenicidade de micoplasmas (MAY & BROWN, 2018).

1.3.1 Estrutura e composição de genomas de *M. hyopneumoniae* e *M. flocculare*

Até 31/05/2022, 26 linhagens de *M. hyopneumoniae* tiveram seus genomas sequenciados e disponibilizados (Tabela 2) foram disponibilizados na base de dados GenBank. Apesar das linhagens com genomas sequenciados divergirem em questão de virulência, como a diminuição na capacidade de adesão aos cílios observada para linhagem J de *M. hyopneumoniae* (ZHANG; YOUNG; ROSS, 1995, 1994; ZIELINSKI & ROSS, 1990, 1993), estudos genômicos comparativos demonstraram uma similaridade de 88% dos

repertórios de genes entre diferentes linhagens (SIQUEIRA *et al.*, 2013; VASCONCELOS *et al.*, 2005).

No caso de *M. flocculare* oito genomas completos sequenciados estão disponíveis na base de dados GenBank. A Tabela 3 apresenta informações sobre os genomas disponíveis.

Tabela 2. Linhagens de *M. hyopneumoniae* com genomas sequenciados e depositados na base de dados GenBank.

Linhagem	Patogenicidade[1]	Referência[2]	Número de acesso Genbank; linhagem ATCC®	Tamanho do genoma (pb)	G + C (%)	CDS	Ano
11	+	(KAMMINGA *et al.*, 2017)	GCA_002193015.1; ATCC®25095™	898.117	28,70	644	2017
98	NE	NP	GCA_013412725.1	880.620	28,60	625	2020
168	+	(LIU *et al.*, 2011)	GCA_000183185.1	925.576	28,50	671	2010
168-L	-	(LIU *et al.*, 2013)	GCA_000400855.1	921.093	28,50	673	2013
232	+	(MINION *et al.*, 2004)	GCA_000008405.1	892.758	28,60	649	2004
7422	+	(SIQUEIRA *et al.*, 2013)	GCA_000427215.1	898.495	28,50	641	2013
7448	+	(VASCONCELOS *et al.*, 2005)	GCA_000008225.1	920.079	28,50	668	2005
ES-2	NE	NP	GCA_004768725.1	956.514	28,40	680	2019
ES-2L	NE	NP	GCA_013402755.1	918.900	28,50	654	2020
F7.2C	+	(TRUEEB *et al.*, 2019)	GCA_007923985.1	894.983	28,60	641	2019
J	-	(VASCONCELOS *et al.*, 2005)	GCA_000008205.1; ATCC®25934™	897.405	28,50	640	2005
KM014	+	(HAN *et al.*, 2017)	GCA_002257505.1	964.503	28,40	900	2017
LH	+	(XIE *et al.*, 2021)	GCA_021383865.1	920.587	28,50	693	2021
MHP650	NE	NP	GCA_009832175.1	903.416	28,60	616	2020
MHP653	NE	NP	GCA_009831945.1	892.502	28,50	625	2020
MHP679	NE	NP	GCA_009832125.1	901.315	28,60	641	2020
MHP682	NE	NP	GCA_009831895.1	905.968	28,60	635	2020
MHP691	NE	NP	GCA_009832085.1	897.976	28,50	625	2020
MHP694	NE	NP	GCA_009831905.1	867.508	28,60	600	2020
MHP696	NE	NP	GCA_009832035.1	874.552	28,60	617	2020
MHP699	NE	NP	GCA_009831855.1	918.878	28,50	641	2020
MHP709	NE	NP	GCA_009832075.1	875.793	28,60	619	2020
NCTC10127	NE	NP	GCA_900660565.1	960.532	28,53	687	2019
TB1	+	(QIU *et al.*, 2019)	GCA_002213485.1	909.064	28,70	602	2017
UFV01	NE	NP	GCA_022793035.1	909.816	28,40	674	2019
UFV02	NE	NP	GCA_022793015.1	959.419	28,50	709	2019

[1]Patogênica (+); não patogênica (-); não encontrada (NE).

[2]Não publicada (NP).

Fonte: https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/Mesomycoplasma%20hyopneumoniae. Data de acesso: 31/05/2022.

Tabela 3. Linhagens de *M. flocculare* com genomas sequenciados e depositados na base de dados GenBank.

Linhagem	Referência[1]	Número de acesso Genbank; linhagem ATCC®	Tamanho do genoma (pb)	G + C %	CDS	Ano
ATCC 27716	(SIQUEIRA *et al.*, 2013)	GCA_000367185.1; ATCC®27716™	763.948	28,90	551	2013
MF11	NP	GCA_009831495.1	765.689	29,50	569	2020
MF18	NP	GCA_009832185.1	758.154	29,00	540	2020
MF22	NP	GCA_009832015.1	752.968	29,00	538	2020
MF29	NP	GCA_009831965.1	777.569	28,90	558	2020
MF30	NP	GCA_009832165.1	733.720	29,10	530	2020
MF33	NP	GCA_009832145.1	756.448	29,00	543	2020
Ms42	(CALCUTT *et al.*, 2015)	GCA_000815065.1; ATCC®27399™	778.866	29,00	575	2015

[1]Não publicada (NP).
Fonte: https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/Mesomycoplasma%20flocculare. Data de acesso: 31/05/2022.

M. hyopneumoniae e *M. flocculare* possuem um cromossomo único circular com tamanhos que variam de 733.720 pb a 964.503 pb, e conteúdo de G + C de aproximadamente 28% (Tabelas 2 e 3). A quantidade de sequências codificadoras de DNA (SCD) entre as linhagens e espécie variam entre 528 e 691 entre as espécies e linhagens (tabela 2 e 3), e, apesar destas diferenças as CDSs (sequências de DNA codificadoras, do inglês, *coding DNA sequences*) constituem 87-88% do genoma de *M. hyopneumoniae* e *M. flocculare* (SIQUEIRA *et al.*, 2013; VASCONCELOS *et al.*, 2005). Estudos genômicos comparativos demonstraram a alta similaridade genética entre *M. hyopneumoniae* e *M. flocculare*, compartilhando mais de 70% dos seus genes (SIQUEIRA *et al.*, 2013), o que explica a proximidade evolutiva destas duas espécies de micoplasmas (Figura 2), já evidenciada em estudos filogenéticos (STEMKE *et al.*, 1992).

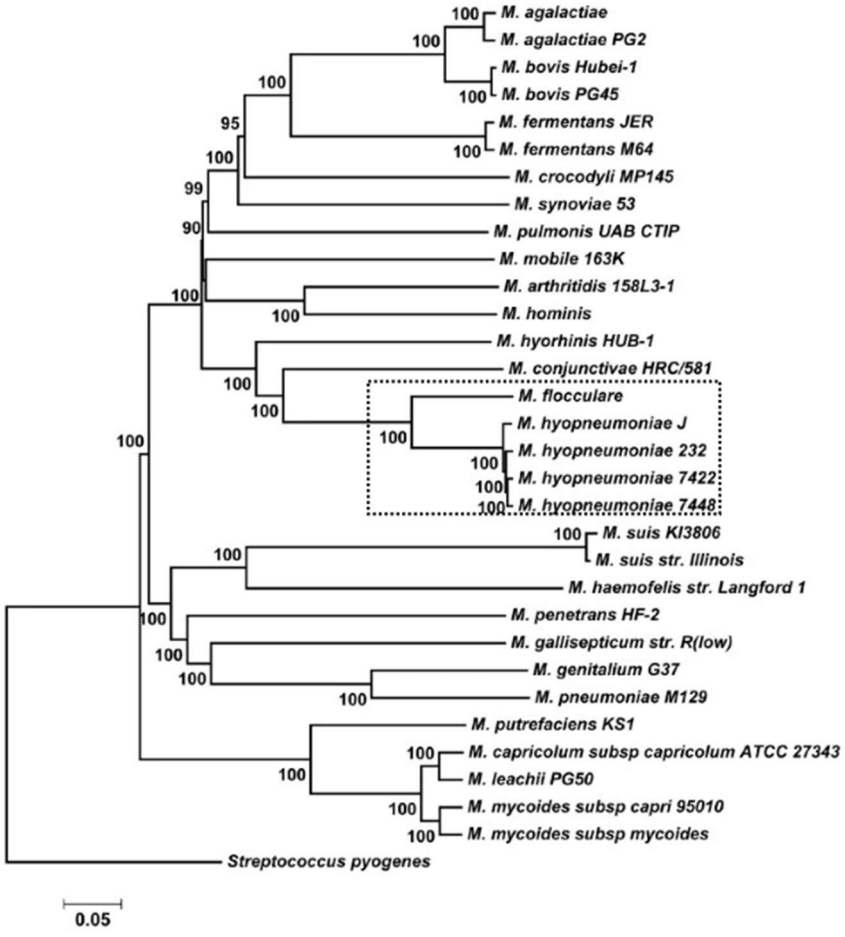

Figura 2. História da evolução de micoplasmas obtida através de uma abordagem filogenômica. *M. flocculare* e linhagens de *M. hyopneumoniae* estão destacadas. Figura adaptada de Siqueira *et al.* (2013).

A organização dos genes em *M. hyopneumoniae* e *M. flocculare* está distribuída em prováveis unidades transcricionais definidas como agrupamentos de fases abertas de leitura (abreviadas como OCs, do inglês *ORF clusters*) ou seja, a ocorrência de duas ou mais CDSs em tandem, observada tanto em estudos *in silico* quanto *in vitro*, para mais de 90% dos genes preditos destas espécies (SIQUEIRA *et al.*, 2013, 2014; SIQUEIRA; SCHRANK; SCHRANK, 2011). Apesar de um genoma reduzido, Vasconcelos *et al.* (2005) identificaram diversos genes linhagem-específicos e com uma composição heterogênea entre as linhagens 232, 7448 e J de *M. hyopneumoniae* (232, 7448 e J). Os rearranjos e inversões intraespecíficos observados, resultaram em agrupamentos de genes linhagem-específicos, o que poderia estar relacionada com a patogênese das linhagens de *M. hyopneumoniae* estudadas. Tais rearranjos também foram observados na comparação entre os genomas de *M. hyopneumoniae* e *M. flocculare* (SIQUEIRA *et al.*, 2013). Apesar destas diferenças, 78% da organização de OCs é totalmente ou parcialmente conservada.

Os micoplasmas são bactérias extremamente fastidiosas e necessitam de nutrientes provenientes do meio de cultura ou hospedeiro para a sobrevivência. Não é de se surpreender que quase metade dos genes codificado são de proteínas de membrana em *M. hyopneumoniae* (292 proteínas, 44,4%) e *M. flocculare* (277 proteínas, 47,5%), principalmente transportadores e enzimas para a degradação de ácidos nucleicos a fim de obter precursores de macromoléculas (SIQUEIRA *et al.*, 2013). Proteínas de superfície em *M. flocculare* não compartilhadas com *M. hyopneumoniae* consistem exclusivamente de proteínas hipotéticas. Das proteínas compartilhadas entre as espécies, 40% possuem funções desconhecidas (proteínas hipotéticas) e o restante consiste em proteínas com funções designadas (60%). Muitas das proteínas compartilhadas (46) são associadas a possíveis fatores de virulência, como lipoproteínas e adesinas. Mais informações sobre proteínas de superfície de micoplasmas são descritas abaixo na seção 1.5.

Além disso as taxas de multiplicação já foram relacionadas com a virulência em outros microrganismos e talvez sejam o fator chave na diferença de patogênese entre essas espécies de micoplasmas e de fato, os modelos *in sílico* demonstram que *M. hyopneumoniae* possui repertórios extras de enzimas que talvez favoreçam o crescimento em comparação espécies não patogênicas, como *M. flocculare*. Em relação aos fatores de virulência com base em metabolômica propõe-se que a diferença de patogenicidade em *M. hyopneumoniae* e *M. flocculare* está relacionada a habilidade de usar o glicerol como fonte de carbono, assim

produzindo peróxido de hidrogênio altamente toxico. Esta característica pode estar diretamente envolvida com a citotoxicidade, uma vez que as capacidades metabólicas entre estas duas espécies são semelhantes com exceção do metabolismo de mio-inositol (FERRARINI *et al.*, 2016). Essa diferença pode der uma das explicações do porquê ambas as espécies têm a capacidade de se aderir ao epitélio ciliar traqueal, mas apenas a adesão de *M. hyopneumoniae* causa danos ao tecido.

1.3.2 Estudos transcritômicos e proteômicos comparativos

Estudos transcritômicos fornecem dados referentes à estrutura e abundância de transcritos, fornecendo dados valiosos para compreensão da função de determinados genes e regulação deles. Para a maioria dos micoplasmas é observado que muitos dos genes co-expressos são policistrônicos (SIQUEIRA; SCHRANK; SCHRANK, 2011) como é observado para outros micoplasmas (GÜELL *et al.*, 2009; HIMMELREICH *et al.*, 1997; SIQUEIRA; SCHRANK; SCHRANK, 2011; WALDO III *et al.*, 1999). Estudos globais *in silico* e *in vitro* sugerem que as ORFs dos OCs são co-transcritos, configurando operons por definição. Além disso, foi observado para *M. hyopneumoniae* que 95% dos genes são transcritos em mRNAs longos policistrônicos (SIQUEIRA *et al.*, 2013). Foi ainda demonstrado que a maioria dos genes em micoplasmas do TRS, são expressos em níveis basais (BEIER; SIQUEIRA; SCHRANK, 2018; FRITSCH; SIQUEIRA; SCHRANK, 2015, 2018; GARDNER & MINION, 2010; SIQUEIRA *et al.*, 2013, 2014; SIQUEIRA; SCHRANK; SCHRANK, 2011; WEBER; SANT'ANNA; SCHRANK, 2012), dificultando explicar diferenças em patogenicidade e virulência entre linhagens de *M. hyopneumoniae* com base apenas em diferenças transcricionais. Contudo, foi observado que *M. hyopneumoniae* pode regular os genes em respostas a alterações do ambiente (KAMMINGA *et al.*, 2020; MADSEN *et al.*, 2006a, 2006b, 2008a, 2008b; MUCHA *et al.*, 2020; NI *et al.*, 2019; ONEAL *et al.*, 2008; SCHAFER *et al.*, 2007; SIQUEIRA *et al.*, 2016). Foram ainda identificados pequenos RNAs não codificadores que possivelmente atuam na regulação da expressão gênica em nível pós-transcricional (SIQUEIRA *et al.*, 2014).

Diversos estudos proteômicos foram também realizados por nosso grupo de pesquisa com o intuito de estudar global e funcionalmente os produtos de expressão gênica do genoma de *M. hyopneumoniae* (LEAL *et al.*, 2016; LEAL ZIMMER *et al.*, 2019a; PAES *et al.*, 2017b, 2019; PINTO *et al.*, 2009, 2007; REOLON *et al.*, 2014). Os estudos proteômicos iniciais, ainda baseados em eletroforese em gel bidimensional e espectrometria de massas

em tandem (2DE-MS/MS, do inglês, *two-dimensional gel electrophoresis and tandem-mass spectrometry*), foram prospectivos e complementaram o trabalho de anotação funcional do genoma sequenciado de *M. hyopneumoniae* 7448, ao mesmo tempo em que evidenciaram novas proteínas antigênicas e processamento pós-traducional proteolítico (PINTO *et al.*, 2007). Seguiram-se então estudos comparativos entre as linhagens 7448 (patogênica) e J (não patogênica) de *M. hyopneumoniae*, ainda por 2DE-MS/MS (PAES *et al.*, 2017b; PINTO *et al.*, 2009). Com isso, já foram evidenciadas diferenças fisiológicas entre as duas linhagens associadas ao caráter de patogenicidade. Mais recentemente, os estudos proteômicos comparativos foram expandidos, passando a incluir a espécie aparentada comensal *M. flocculare*, e aprofundados, a partir de abordagens de LC-MS/MS em espectrômetros de massa de maior resolução e sensibilidade. Tais estudos compararam extratos celulares totais (PINTO *et al.*, 2009; REOLON *et al.*, 2014), produtos de secreção das micoplasmas (PAES *et al.*, 2017b) e identificaram alterações nos proteomas celulares e nos secretomas de secreção de células traqueais suínas em resposta ao co-cultivo com estas bactérias (LEAL ZIMMER *et al.*, 2019a; LEAL ZIMMER *et al.*, 2019b). Foi ainda estudada por LC-MS/MS a resposta diferencial a estresses entre as linhagens 7448 e J de *M. hyopneumoniae* (PAES *et al.*, 2019). Todos estes estudos prospectivos e comparativos prévios envolvendo linhagens patogênicas e não patogênicas de *M. hyopneumoniae* e a espécie comensal *M. flocculare* permitiram evidenciar diferenças qualiquatitativas nos repertórios de proteínas associadas a virulência e patogenicidade. Os estudos proteômicos adicionais que compões esta tese deram continuidade a esta linha de investigação em nosso grupo de pesquisa.

1.4 Proteínas de superfície bacterianas e suas interações patógeno-hospedeiro

As membranas citoplasmáticas bacterianas costumam ser compostas por porções igualitárias de fosfolipídeos e proteínas que representam aproximadamente 65-75% e 6-9%, respectivamente. Os fosfolipídeos são compostos por uma porção hidrofóbica (ácidos graxos) e uma porção hidrofílica (glicerol) (SCHUMANN, 2006). A membrana deve manter uma estrutura fluída e liquida para permitir a difusão lateral de suas proteínas e complexos proteicos. Além disso, membranas fluídas possuem uma maior permeabilidade de moléculas pequenas (STRAHL & ERRINGTON, 2017). O arranjamento da bicamada de fosfolipídeos, formando uma barreira hidrofóbica, impede o movimento não controlado de moléculas polares e permite a retenção de metabólitos e proteínas.

As proteínas e complexos proteicos situados na membrana possuem um papel fundamental em diversos processos celulares, que incluem o controle de moléculas, nutrientes e íons através das membranas, secreção de proteínas entre o meio intracelular e extracelular, bem como a participação na sinalização celular e motilidade (RAWLINGS, 2016; STRAHL & ERRINGTON, 2017). As proteínas de membrana podem ser classificadas em duas categorias amplas, (*i*) integrais ou intrínsecas, que estão fortemente ligadas a membrana com um ou mais domínios, como permeases; e (*ii*) periféricas ou extrínsecas, que estão fracamente ou transitoriamente ligadas à membrana, como receptores extracelulares de ligação a solutos (LODISH; BERK; ZIPURSKY, 2000; POETSCH & WOLTERS, 2008; SCHUMANN, 2006). As proteínas de superfície podem também ser classificadas pela função que possuem como: (*i*) proteínas envolvidas na geração e conservação de energia; (*ii*) proteínas envolvidas no transporte de solutos; (*iii*) proteínas envolvidas na translocação de carboidratos; (*iv*) proteínas e complexos proteicos envolvidos na translocação de proteínas para a membrana citoplasmática (SCHUMANN, 2006). Os resíduos de aminoácidos dessas proteínas que estão em contato direto com a porção apolar da membrana, possuem um caráter hidrofóbico, assim como resíduos no interior de proteínas solúveis. Já os resíduos expostos no ambiente aquoso têm um caráter polar ou hidrofílico (SCHUMANN, 2006). Os resíduos expostos as cadeias acil-lipídicas possuem um caráter ainda mais hidrofóbico que os resíduos no interior da membrana, importantes para manter a conformação e inserção correta na bicamada apolar da membrana.

Em bactérias, um quarto a um terço do proteoma predito correspondem a proteínas integrais da membrana. Entretanto, devido à natureza hidrofóbica e hidrofílica das proteínas de membrana as torna difíceis para estudar (LODISH; BERK; ZIPURSKY, 2000; RAWLINGS, 2016; SCHUMANN, 2006; STRAHL & ERRINGTON, 2017). A hidrofobicidade de proteínas de membrana certamente contribui para a sua baixa representação em estudos proteômicos globais (MACHER & YEN, 2007). Por tais motivos, uma abordagem para análise de proteínas de membrana é o enriquecimento prévio desta fração subcelular. Além disto, estratégias como a utilização de diversos detergentes e solventes orgânicos, e utilização de proteases afim de expor domínios de proteínas integrais da membrana (MACHER & YEN, 2007).

O conhecimento e caracterização de proteínas de membrana e proteínas associadas a membrana possui um interesse particular pela interação entre patógeno-hospedeiro,

desenvolvimento de novos fármacos e envolvimento na adesão ao hospedeiro e resistência à antimicrobianos (NICOD; BANAEI-ESFAHANI; COLLINS, 2017; POETSCH & WOLTERS, 2008). Assim como estudos da expressão diferencial de proteínas do hospedeiro, em resposta ao patógeno bacteriano, pode fornecer informações sobre seus mecanismos moleculares subjacentes de patogenicidade e, potencialmente, até mesmo alvos de intervenção farmacológica específicos (NICOD; BANAEI-ESFAHANI; COLLINS, 2017).

1.5 Proteínas de superfície de *M. hyopneumoniae*

Diferentemente de outras bactérias, micoplasmas não possuem parede celular e membrana intracelular, possuindo apenas uma membrana simples, composta basicamente por lipídeos (fosfolipídeos e colesterol) 20-30% (um terço) da massa e proteínas de membrana 60-70% (dois terços) da massa (RAZIN, 1996; RAZIN & HAYFLICK, 2010; ROTTEM & KAHANE, 1993). Atenção especial foi dada ao colesterol, um componente peculiar da membrana de micoplasmas, que não são geralmente encontradas em outras membranas de procariotos. Os lipídeos presentes na membrana se assemelham aqueles semelhantes a outras bactérias, com exceção das grandes quantidades de colesterol requeridos por micoplasmas. A ausência total de parece celular de micoplasmas explica muitas das suas propriedades únicas, como sensibilidade ao choque osmótico e detergentes, e resistência à penicilina (RAZIN & HAYFLICK, 2010).

Por serem dependentes de muitos nutrientes fornecidos pelo hospedeiro ou meio de cultura, micoplasmas possuem uma variedade de transportadores de membrana. Por possuírem apenas uma única membrana celular, a membrana de micoplasmas é esperada ser rica em atividades enzimáticas, bem como possuir transportadores específicos de nutrientes (RAZIN & HAYFLICK, 2010; ROTTEM & KAHANE, 1993). De fato, aproximadamente um terço do proteoma predito de *M. hyopneumoniae* e *M. flocculare* correspondem a proteínas de superfície, que possuem a função de sinalização celular, tráfico de moléculas, adesão celular e modulação da resposta imune (SIQUEIRA *et al.*, 2013).

Micoplasmas possuem alguns antígenos de superfície, como proteínas de membrana, lipoproteínas, glicolipídios e lipopolissacarídeos (RAZIN, 1996). Algumas destas proteínas de membrana sofrem variação antigênica. A presença de determinantes de virulência na superfície de espécies e linhagens não patogênicas (*M. flocculare* e *M. hyopneumoniae* J, *M. hyopneumoniae* 168-L), demonstra que a patogenicidade pode ser dependente dos níveis de

expressão, processamento proteolítico diferencial, além das variações do número de repetições de aminoácidos entre as proteínas ortólogas, gerando variantes funcionais e/ou antigênicos (FERREIRA & DE CASTRO, 2007; LIU *et al.*, 2013; SIQUEIRA *et al.*, 2013; VASCONCELOS *et al.*, 2005). Um exemplo dessa variação foi observado para as proteínas de membrana P97 (DE CASTRO *et al.*, 2006; DEUTSCHER *et al.*, 2010; HSU & MINION, 1998a, 1998b; MINION; ADAMS; HSU, 2000; SIQUEIRA *et al.*, 2013; WILTON *et al.*, 1998), P76, P95, P146, P216 e diversas proteínas não caracterizadas (DE CASTRO *et al.*, 2006; SIQUEIRA *et al.*, 2013). Além disso, a maioria das diferenças espécie-especificas não estão relacionadas a genes com função conhecida e/ou envolvidos com a patogenicidade. As maiores diferenças estão relacionadas com proteínas hipotéticas, de transporte, utilização do mio-inositol e à cópia adicional da p97.

Dentre as proteínas de superfície de micoplasmas, têm especial destaque as lipoproteínas e as adesinas, por serem consideradas potenciais fatores de virulência e determinantes de patogenicidade (FERREIRA & DE CASTRO, 2007). Surpreendentemente, quando se compara *M. hyopneumoniae* e *M. flocculare*, a maior parte das lipoproteínas e adesinas descritas são comuns às duas espécies, apesar da diferença de patogênese entre elas (FERRARINI *et al.*, 2016; SIQUEIRA *et al.*, 2013; VASCONCELOS *et al.*, 2005). Por exemplo, *M. flocculare* possui ortólogas de todas as proteínas relacionadas à adesão de *M. hyopneumoniae*, com exceção de uma cópia da adesina P97 e uma da P102. A organização genômica de P97-cópia 2 e P97-*like* é similar entre as espécies também no que diz respeito à organização em OCs. Uma diferença entre *M. hyopneumoniae* e *M. flocculare* é observada quanto à organização dos genes das adesinas P216, P159 e P60, que são extremamente conservadas nas linhagens de *M. hyopneumoniae* 232, 7422, 7448 e 168, mas apresentam outra distribuição do genoma de *M. flocculare*, devido a rearranjos e inversões (SIQUEIRA *et al.*, 2013; VASCONCELOS *et al.*, 2005). Ainda, algumas diferenças menores entre ortólogos de adesinas são observadas, como a ausência das repetições R1 e R2 na adesina P97-*like* de *M. flocculare* (SIQUEIRA *et al.*, 2013). Outras diferenças em domínios de ortólogas de adesinas e outras proteínas de superfície de *M. hyopneumoniae* e *M. flocculare* foram depois identificadas, inclusive algumas determinantes de diferenças imunológicas entre elas (LEAL *et al.*, 2016).

2. JUSTIFICATIVAS

As perdas econômicas devido à PES estão relacionadas principalmente a gastos com medicamentos para o tratamento, vacinação, diminuição da performance e aumento da mortalidade devido a infecções secundárias (MAES *et al.*, 2018). Apesar da mortalidade causada por infecções de *M. hyopneumoniae* ser considerada baixa, as perdas econômicas relacionadas são altas. A PES leva a perdas econômicas significativas na suinocultura, principalmente pela redução da conversão alimentar e consequente diminuição do ganho de peso médio diário, maior tempo para obtenção do peso para abate, gastos com o tratamento e controle, e mortalidade relacionada a infecções secundárias (HOLST; YESKE; PIETERS, 2015; TAO *et al.*, 2019). Além disso, a vacinação adiciona mais dificuldade relacionada ao diagnóstico de animais infectados, dificulta a contenção dos casos em granjas, uma vez que dificulta a separação dos animais doentes daqueles com anticorpos provenientes da vacinação. Dessa maneira, pesquisas adicionais são necessárias para discriminar entre suínos infectados e vacinados (BAI *et al.*, 2018).

Como micoplasmas são organismos que possuem diversas estratégias para evitar a resposta imune de seus hospedeiros, uma completa compreensão de mecanismos relacionados com o desenvolvimento da doença e virulência pode fornecer informações fundamentais para combater este patógeno. Além disto, a resistência intrínseca de micoplasmas a antibióticos que atuam na parede celular, o aumento da resistência a antimicrobianos e o baixo sucesso no desenvolvimento de vacinas efetivas, evidenciam a necessidade de maior compreensão dos fatores de virulência associados a doenças. Podendo, assim, fornecer alternativas de possíveis alvos terapêuticos e diagnóstico para o controle e manejo destas infecções. Desta forma, o conhecimento dos fatores relacionados com a virulência se torna importante e fornece possíveis alvos para o desenvolvimento de vacinas e fármacos a fim de controlar as infecções causadas por micoplasmas (CITTI & BLANCHARD, 2013; KANDAVELMANI & PIRAMANAYAGAM, 2019).

Nesse aspecto, estudos de proteínas de superfície de micoplasmas podem ser uma estratégia interessante para a identificação de fatores de patogenicidade e modulação da resposta imune do hospedeiro. Principalmente por estarem em contato direto com as células do hospedeiro, a comparação entre o repertório diferencial de proteínas de superfície entre as linhagens e espécies patogênicas e não patogênicas podem trazer diversas contribuições necessárias para a elucidação dos diferentes mecanismos utilizados por micoplasmas do TRS no desenvolvimento ou ausência da doença. Assim, essa tese se propõe a identificar os

proteomas de superfície de espécies/linhagens de micoplasmas consideradas patogênicas e não patogênicas, a fim de identificar determinantes de virulência ou evidenciar processos moleculares determinantes para moldar a superfície bacteriana e suas variações nos proteomas de superfície.

3. OBJETIVOS

3.1 Objetivo geral

O objetivo geral deste trabalho é descrever e comparar qualitativamente e quantitativamente os proteomas de superfície de *M. hyopneumoniae* e *M. flocculare*, para a identificação de proteínas associadas à patogenicidade, e avaliar e comparar o processamento proteolítico de adesinas conhecidas destas duas espécies.

3.2 Objetivos específicos

3.2.1. Caracterizar qualitativamente e quantitativamente os repertórios de superfície de *M. hyopneumoniae* 7448, *M. hyopneumoniae* J e *M. flocculare*;

3.2.2. Comparar os repertórios de proteínas de superfície de *M. flocculare* e das linhagens 7448 e J de *M. hyopneumoniae*;

3.2.3. Identificar diferenças qualitativas e quantitativas nos repertórios de proteínas de superfície entre *M. hyopneumoniae* 7448 e linhagens/espécies consideradas não patogênicas que possam estar relacionadas ou não à patogenicidade;

3.2.4. Fornecer evidências adicionais do processamento proteolítico de adesinas na superfície de linhagens de *M. hyopneumoniae* e *M. flocculare* com base nos resultados de espectrometria de massas;

3.2.5. Avaliar e comparar o processamento proteolítico das cinco adesinas mais abundantes no proteoma de superfície em *M. hyopneumoniae* 7448 e seus respectivos ortólogos em *M. hyopneumoniae* J e *M. flocculare*.

4. MATERIAIS E MÉTODOS E RESULTADOS

Os materiais e métodos e os resultados estão organizados em duas seções, ambas compostas por artigos científicos publicados durante o período do doutorado. A seção 4.1, é composta pela análise proteômica comparativa de duas linhagens, 7448 e J, de *M. hyopneumoniae* e *M. flocculare*. A seção 4.2 é composta por análises de processamento proteolítico diferencial das proteínas de superfície relacionadas à adesão mais abundantes em *M. hyopneumoniae* 7448 e suas respectivas ortólogas em *M. hyopneumoniae* J e *M. flocculare*.

4.1 Análise comparativa dos proteomas de superfície e citoplasmáticas de duas linhagens de *M. hyopneumoniae* e de *M. flocculare*

O artigo que constitui esta seção, intitulado *Comparative proteomics of two* Mycoplasma hyopneumoniae *strains and* Mycoplasma flocculare *identified potential porcine enzootic pneumonia determinants,* foi publicado na revista Virulence (https://doi.org/10.1080/21505594.2018.1499379). Os resultados estão associados aos objetivos 3.2.1, 3.2.2 e 3.2.3 desta tese. O artigo possui a primeira autoria compartilhada entre a autora Lais Del Prá Netto Machado e a Dra. Jéssica Andrade Paes. As contribuições dos coautores estão descritas abaixo. O material suplementar (*Supplemental material*) associado está disponível pelo link https://www.tandfonline.com/doi/suppl/10.1080/21505594.2018.1499379?scroll=top

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Contribuição dos autores:

LDPNM e JAP compartilharam a primeira autoria. LDPNM: delineamento experimental, execução da análise do proteoma de superfície de *M. hyopneumoniae* e *M. flocculare*, redação do manuscrito; JAP: delineamento experimental, execução da análise do proteoma citoplasmático de *M. hyopneumoniae* e *M. flocculare*, redação do manuscrito; FMAL: auxílio nas análises de LC-MS/MS, discussão dos resultados; SNM: auxílio na preparação de amostras para LC-MS/MS; HM e JRB: auxílio nas análises de LC-MS/MS, discussão dos resultados, revisão do manuscrito; HBF: delineamento experimental, análise e discussão de resultados, e revisão do manuscrito.

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Comparative proteomics of two *Mycoplasma hyopneumoniae* strains and *Mycoplasma flocculare* identified potential porcine enzootic pneumonia determinants

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ABSTRACT

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* are genetically similar bacteria, which coinhabit the porcine respiratory tract. These mycoplasmas share most of the known virulence factors, but, while *M. hyopneumoniae* causes porcine enzootic pneumonia (PEP), *M. flocculare* is a commensal species. To identify potential PEP determinants and provide novel insights on mycoplasma-host interactions, the whole cell proteomes of two *M. hyopneumoniae* strains, one pathogenic (7448) and other non-pathogenic (J), and *M. flocculare* were compared. A cell fractioning approach combined with mass spectrometry (LC-MS/MS) proteomics was used to analyze cytoplasmic and surface-enriched protein fractions. Average detection of ~ 50% of the predicted proteomes of *M. hyopneumoniae* 7448 and J, and *M. flocculare* was achieved. Many of the identified proteins were differentially represented in *M. hyopneumoniae* 7448 in comparison to *M. hyopneumoniae* J and *M. flocculare*, including potential PEP determinants, such as adhesins, proteases, and redox-balancing proteins, among others. The LC-MS/MS data also provided experimental validation for several genes previously regarded as hypothetical for all analyzed mycoplasmas, including some coding for proteins bearing virulence-related functional domains. The comprehensive proteome profiling of two *M. hyopneumoniae* strains and *M. flocculare* provided tens of novel candidates to PEP determinants or virulence factors, beyond those classically described.

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Introduction

The identification and characterization of virulence factors is of upmost relevance to discover new targets for the development of diagnostic methods, therapeutic drugs, and vaccines [1]. However, the multifactorial nature of pathogenicity poses difficulties to identify disease-related proteins and mechanisms in pathogenic species. Comparisons between virulent and avirulent strains of a pathogenic species and/or two closely-related species that coinhabit the same host species, being one pathogenic and the other a commensal, are expected to provide valuable information on determinants of pathogenic/commensal ways of life.

Among the mycoplasmas that coinhabit the swine respiratory tract, there are two interesting species for comparative studies: the pathogenic *Mycoplasma hyopneumoniae* and the commensal *Mycoplasma flocculare* [2]. *M. hyopneumoniae* adheres to the host respiratory epithelium and causes the porcine enzootic pneumonia (PEP). *M. flocculare* also adheres to porcine respiratory epithelium and can be isolated from normal and pneumonic lungs. This species is usually regarded as non-pathogenic [3,4], although it is considered by some authors an opportunistic pneumonic pathogen in coinfections with *M. hyopneumoniae* [5]. Despite the pathogenic nature of *M. hyopneumoniae*, there are some strains that vary in their virulence levels, or even are avirulent, such as *M. hyopneumoniae* J, which has reduced adhesion capacity to porcine cilia [6]. Comparisons between the genomes of *M. hyopneumoniae* pathogenic and non-pathogenic strains (7448 and J, respectively) revealed no extensive genomic differences [7]. Moreover, previous comparative phylogenetic and phylogenomic studies provided evidences of

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Supplementary data for this article can be accessed here.

the close relationship of *M. hyopneumoniae* and *M. flocculare* [7–9], which share most of the known virulence-related genes [10]. The differences between *M. hyopneumoniae* and *M. flocculare* include the absence, in *M. flocculare*, of the *glpO* gene, related to *M. hyopneumoniae* hydrogen peroxide generation and cytotoxicity [11,12], and differential domains between orthologs from the P97 family of adhesins and from other surface proteins [13]. However, 90% of *M. flocculare* predicted surface proteins are shared with *M. hyopneumoniae* [9], and the observed genomic differences between *M. hyopneumoniae* strains, and between *M. hyopneumoniae* and *M. flocculare* so far do not fully explain their differential phenotypes of virulence/pathogenicity.

Differential expression of ortholog genes may also contribute to differences in pathogenicity or virulence level between *M. hyopneumoniae* strains or between *M. hyopneumoniae* and *M. flocculare*. However, previous comparative transcriptomic studies between *M. hyopneumoniae* and *M. flocculare* [14] failed to find differences in the relative transcription levels for most genes. On the other hand, pioneer proteomic studies, have provided evidences of differential protein abundance and post-translational processing between *M. hyopneumoniae* pathogenic (7448 and 7422) and non-pathogenic (J) strains [15]. Moreover, a recent comparative proteomics study between *M. hyopneumoniae* and *M. flocculare* secreted proteins revealed several virulence-related differences between these mycoplasma species [16]. This study showed that the *M. hyopneumoniae* secretome included several virulence-related proteins, like adhesins, transporters, nucleases and uncharacterized proteins bearing virulence-related functional domains, not found in the *M. flocculare*, secretome. Overall, these previous studies indicate the necessity of further and more comprehensive comparative proteomic studies, to deeply investigate possible pathogenicity or virulence-related differences at the protein level.

Here, the whole cell proteomes of *M. hyopneumoniae* strains 7448 (pathogenic) and J (non-pathogenic), and *M. flocculare* were compared by a mass spectrometry (MS)-based approach to identify differences in protein abundance associated with pathogenicity or virulence. Mycoplasma cells were fractioned into cytoplasmic- and surface-enriched protein fractions and their protein contents were analyzed by high-resolution and high-sensitivity MS. Several significant differences among *M. hyopneumoniae* strains and *M. flocculare* proteomes were depicted, and their biological significance for mycoplasma-host interactions, for virulence and PEP determination are discussed.

Results

MS-based identification of proteins from M. hyopneumoniae 7448 and J, and M. flocculare

LC-MS/MS analyzes of proteins from soluble and insoluble fractions identified overall totals of 344 out of 695 (~ 50%), 343 out of 672 (~ 51%), and 315 out of 581 (~ 54%) protein species from *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare*, respectively. Detailed peptide and protein identification data are presented in Supplementary Tables 1 and 2, respectively. Around 70% (242) of the detected proteins were shared between *M. hyopneumoniae* 7448, J, and *M. flocculare* (Figure 1(A), Supplementary Table 3A). The average peptide coverage for both *M. hyopneumoniae* and *M. flocculare* identified proteins was ~ 40% in soluble fraction and ~ 20% in insoluble fraction. The calculated zero false discovery rates (FDR) for the proteins and peptides of all samples validated all MS/MS results.

Considering the detected proteins in soluble fractions, 287, 239, and 286 proteins were identified in *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare* samples, respectively. Most (188) of the identified proteins in this fraction type (considering ortholog ones) were shared between the two *M. hyopneumoniae* strains, and *M. flocculare* (Figure 1(B), Supplementary Table 3B). Considering the number of detected proteins in insoluble fractions, 267 proteins were identified for *M. hyopneumoniae* 7448, 304 proteins, for *M. hyopneumoniae* J, and 179 proteins, for *M. flocculare*. Most (135) proteins identified in these fractions are shared among all analyzed mycoplasmas (Figure 1(C), Supplementary Table 3C).

Overall, these preliminary comparisons indicated qualitative differences among the proteomes of *M. hyopneumoniae* 7448 and J, and *M. flocculare*. Some of these differences between pathogenic and non-pathogenic mycoplasmas may be associated with pathogenicity.

Enrichment of surface-related proteins in insoluble extracts of M. hyopneumoniae *7448 and J, and M. flocculare*

The identified protein repertoires from soluble and insoluble protein extracts were compared to confirm the enrichment of the insoluble fraction with surface proteins. *In silico* subcellular localization predictions for proteins identified in insoluble and soluble extracts are detailed in Supplementary Table 4. Higher numbers of proteins predicted as surface proteins were identified in the insoluble fractions of *M. hyopneumoniae* strains 7448 (111;

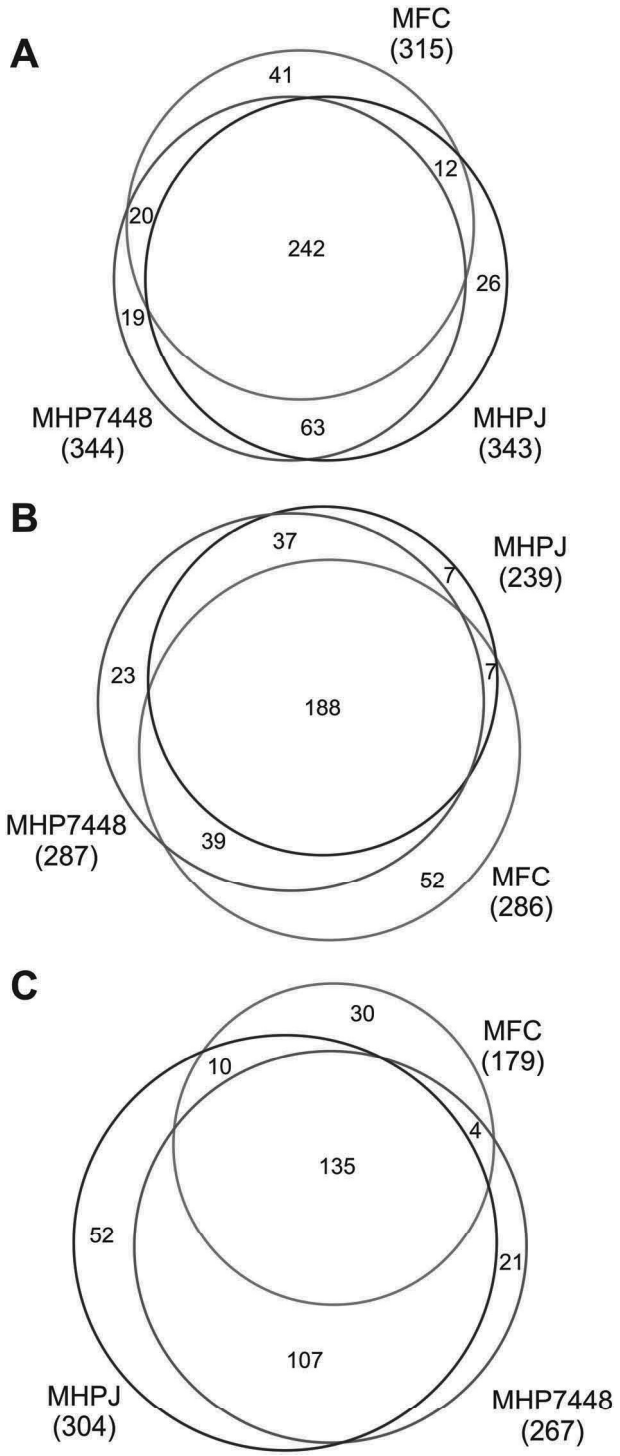

Figure 1. Overview of proteins identified in *M. hyopneumoniae* 7448 (MHP7448) and J (MHPJ), and *M. flocculare* (MFC) samples. (A) Total proteins. (B) Proteins detected in soluble fractions. (C) Proteins detected in insoluble fractions. Overall numbers of proteins identified for each sample between parentheses. Numbers of proteins exclusively detected in each sample or shared between them are indicated within the Venn diagrams.

41.6%) and J (116; 38.2%), and for *M. flocculare* (84; 46.9%) in comparison to those identified in the corresponding soluble extracts (~ 25%). Conversely, most proteins identified in the soluble fractions of *M. hyopneumoniae* 7448 (168; 58.5%), *M. hyopneumoniae* J (144; 60.3%) and *M. flocculare* (161; 56.3%) were predicted as cytoplasmic proteins. Overall, these preliminary results showed qualitative

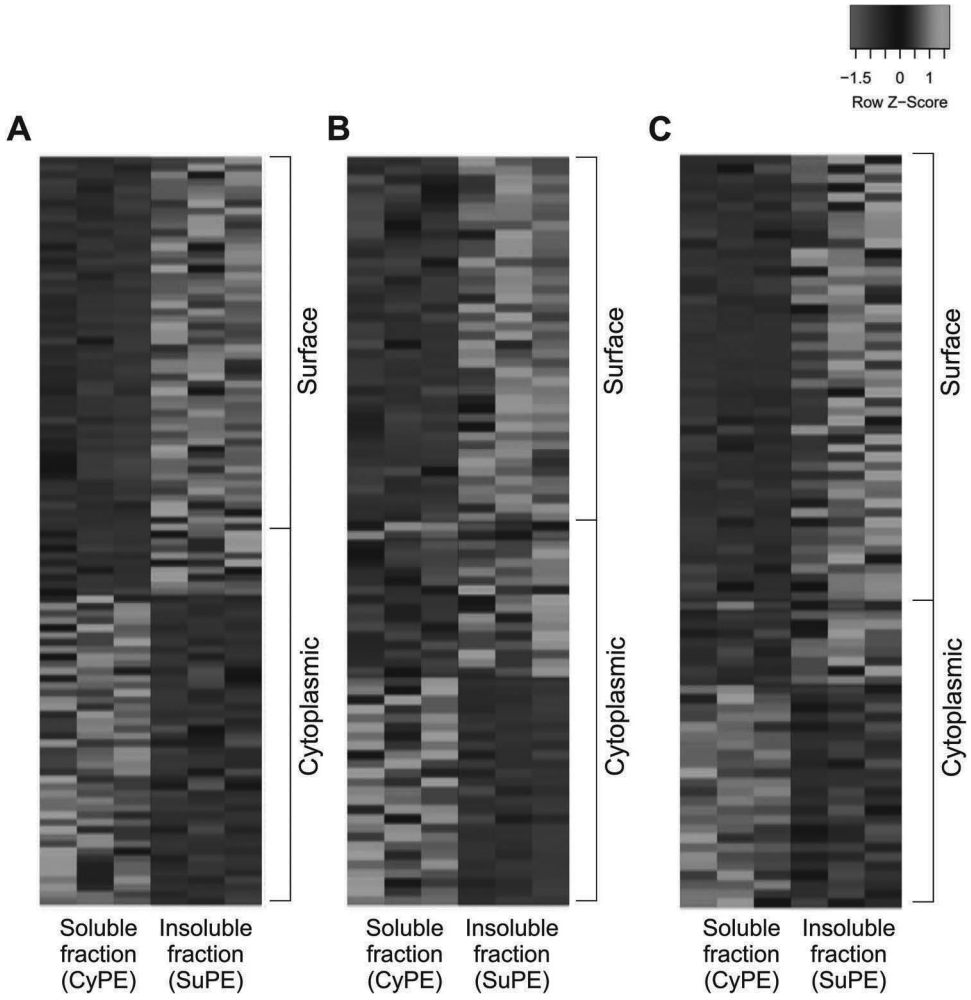

Figure 2. Heatmaps showing the enrichment of surface-related proteins in insoluble extracts of (A) *M. hyopneumoniae* 7448, (B) *M. hyopneumoniae* J, and (C) *M. flocculare*. In each heat map, all shared proteins showing statistically significant abundances ($p < 0.05$) between soluble (CyPE) and insoluble (SuPE) fractions are represented (red, low abundance; green, high abundance). Distribution of surface and cytoplasmic-predicted proteins are indicated on the right. NSAF values, converted in Z-scores, were used to quantify relative differences in protein abundance, and the *t*-test was applied to determine statistically significant differences between shared proteins.

differences between extracts that demonstrate the enrichment of surface proteins in insoluble extracts of *M. hyopneumoniae* (7448 and J) and *M. flocculare*.

Additionally, to verify the quantitative enrichment of surface proteins in insoluble protein extracts, the differential abundance of shared proteins between soluble and insoluble fractions were assessed using NSAF values. For *M. hyopneumoniae* 7448, 125 out of 210 proteins (59%) were differentially abundant between these fractions, while 93 out of 200 (46%) and 89 out of 150 (59%) were differentially abundant for *M. hyopneumoniae* J, and for *M. flocculare*, respectively (Supplementary Table 4). Among overrepresented proteins, there was an evident enrichment of surface-predicted proteins in insoluble fractions for all analyzed mycoplasmas, while cytoplasmic proteins were more abundant in soluble fractions (Figure 2).

Overall, the repertoires of differential proteins demonstrated the clear enrichments of surface proteins and cytoplasmic proteins in the analyzed insoluble and soluble fractions, respectively. Therefore, from now on these mycoplasma soluble and insoluble fractions will be treated as cytoplasmic-enriched protein extracts (CyPE) and surface-enriched protein extracts (SuPE), respectively.

Differences between the whole-cell protein contents of M. hyopneumoniae 7448 and J, and M. flocculare

The whole-cell proteome (including proteins detected in both CyPE and SuPE) of *M. hyopneumoniae* 7448 was

qualitatively and quantitatively analyzed and compared to those from *M. hyopneumoniae* J and *M. flocculare*. Qualitative comparisons were based on presence/absence of detected ortholog proteins, while quantitative comparisons were performed between ortholog proteins shared between *M. hyopneumoniae* 7448 and J, and between *M. hyopneumoniae* 7448 and *M. flocculare*.

In the whole-cell proteomes, 39 and 82 proteins were exclusively detected in *M. hyopneumoniae* 7448 samples in comparison to *M. hyopneumoniae* J and *M. flocculare*, respectively (see Figure 1). Separately analyzing the CyPEs, 62 and 60 proteins were found exclusively in *M. hyopneumoniae* 7448 samples in comparison to *M. hyopneumoniae* J and *M. flocculare*, respectively. On the other hand, 25 and 128 proteins were *M. hyopneumoniae* 7448-exclusive in comparison to *M. hyopneumoniae* J and *M. flocculare*, respectively, considering only SuPE samples.

Quantitative analyzes were performed with shared proteins between samples based on emPAI values (Supplementary Table 5). *M. hyopneumoniae* 7448 and J strains shared 305 proteins, while *M. hyopneumoniae* 7448 and *M. flocculare* shared 262. Among the proteins shared between *M. hyopneumoniae* 7448 and J strains, 78 proteins were differentially abundant. In comparison to *M. hyopneumoniae* J, 25 CyPE proteins and 18 SuPE proteins were overrepresented in *M. hyopneumoniae* 7448 samples. The differences in abundance ranged from 1.5 to 7.3-fold. Only some SuPE proteins (35 proteins) were underrepresented in *M. hyopneumoniae* 7448 samples in comparison to the J strain.

Quantitative comparisons between *M. hyopneumoniae* 7448 to *M. flocculare* found 79 proteins differentially abundant. Twenty CyPE and 44 SuPE proteins were overrepresented in *M. hyopneumoniae* 7448 samples, with differences in abundance ranging from 1.7 to 63-fold. Nine CyPE and 6 SuPE proteins were underrepresented in *M. hyopneumoniae* 7448 samples in this comparison. Among these differentially abundant proteins from *M. hyopneumoniae* 7448 and *M. flocculare*, only 9 presented significant abundance differences in both CyPE and SuPE. Of these proteins seven were overrepresented in both protein extracts of *M. hyopneumoniae* 7448. The other 2 represent cases of differential enrichment between the subcellular fractions in these two species. An aminopeptidase was overrepresented in the CyPE and underrepresented in the SuPE, in *M. hyopneumoniae* 7448, and vice-versa, in *M. flocculare*. Conversely, an uncharacterized protein (MHP7448_0356), was underrepresented in the CyPE and overrepresented in the SuPE, in *M. hyopneumoniae* 7448, and vice-versa, in *M. flocculare*.

Overall, these results showed important qualitative and quantitative differences between *M. hyopneumoniae* 7448 and J strains, between *M. hyopneumoniae* 7448 and *M. flocculare* regarding whole-cell proteomes. These differences can be associated with the differential pathogenic and non-pathogenic natures of these mycoplasmas and may point out some potential PEP determinants as described in the next sections.

Potential PEP determinants differentially represented in M. hyopneumoniae 7448

Differential proteins between *M. hyopneumoniae* 7448 and its non-pathogenic counterparts were assumed to be potential PEP determinants. This assumption was validated by the fact that, among these differential proteins, there were many virulence-related proteins previously described in the literature, like adhesins, proteases, redox balancing protein, and membrane transporters. The observed qualitative differences are graphically represented in Figure 3, and quantitative differences are presented in Table 1.

Qualitative comparisons revealed that most of the differential proteins were detected in both CyPE and SuPE samples for all analyzed mycoplasmas (see Supplementary Table 2). However, some proteins were exclusively detected in only one subcellular fraction, as follows. Methionine aminopeptidase was exclusively found in the CyPE from *M. hyopneumoniae* 7448 (MHP7448_0173), *M. hyopneumoniae* J (MHJ_0169) and, *M. flocculare* (MFC_0210). The XAA-PRO aminopeptidase was exclusively detected in the CyPE in *M. flocculare*, while in both *M. hyopneumoniae* strains it was detected in both CyPE and SuPE. The neutrophil activating factor, which is involved in oxidative stress, was exclusively detected in *M. hyopneumoniae* 7448 CyPE samples (MHP7448_0457). Most of the detected membrane transporters protein species were found only in SuPE or in both CyPE and SuPE samples from all analyzed mycoplasmas. As expected, those membrane transporters shared between CyPE and SuPE samples were mostly enriched in the SuPE samples (see Supplementary Table 4).

Considering the proteins detected in both CyPE and SuPE, several quantitative differences were observed involving the *M. hyopneumoniae* 7448 adhesin repertoire in comparison to those of the non-pathogenic counterparts. In comparison to *M. hyopneumoniae* J, the P97-like (MHP7448_0272) and MgpA-like (MHP7448_0005) adhesins were overrepresented in *M. hyopneumoniae* 7448 CyPE. On the other hand, the P95 (MHP7448_0099) and P102-copy 1 (MHP7448_0199) adhesins, were underrepresented in *M. hyopneumoniae* 7448 SuPE. Comparisons between *M. hyopneumoniae* 7448 and *M. flocculare* adhesins, showed that the P65 adhesin (MHP7448_0656) was overrepresented in *M. hyopneumoniae* 7448 in both

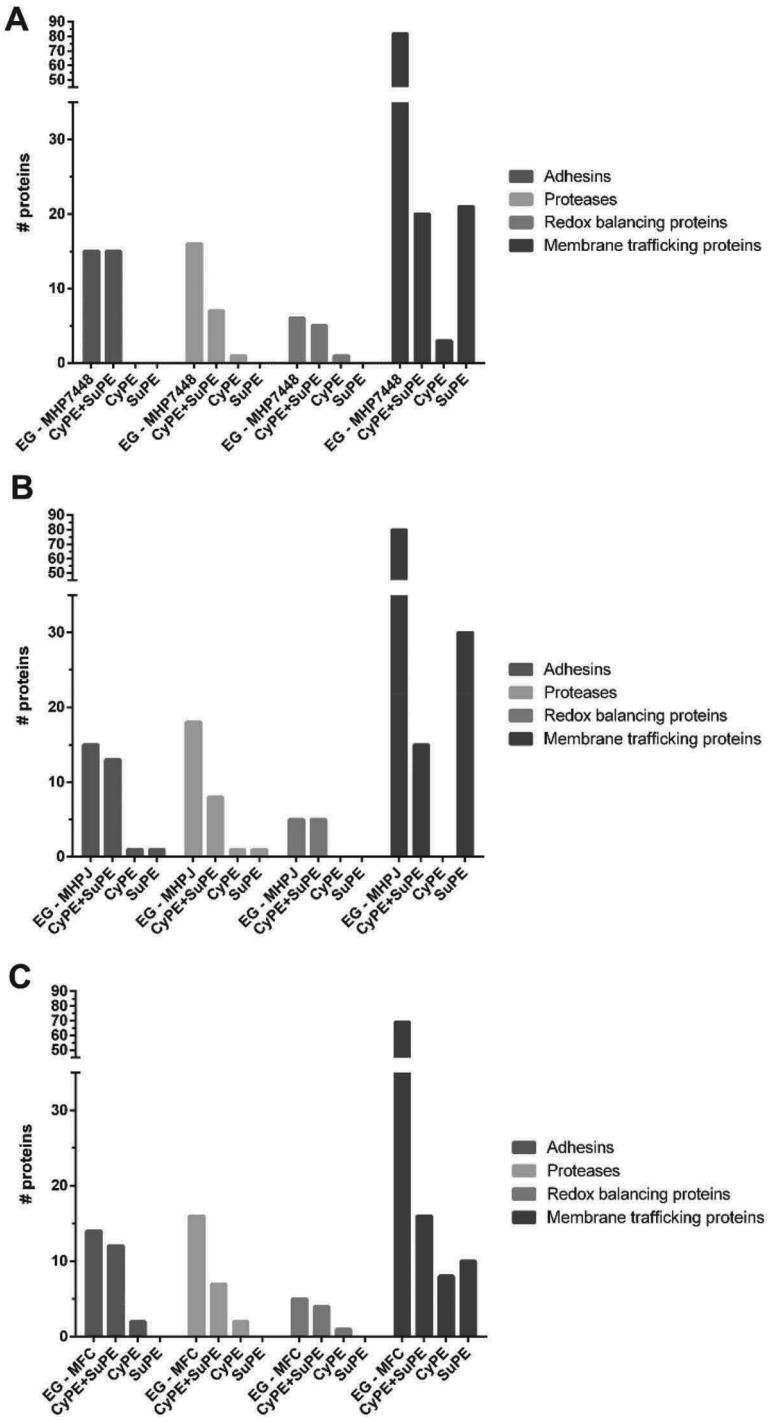

Figure 3. Qualitative differences in potential PEP determinants detected in CyPE and/or SuPE of (A) *M. hyopneumoniae* 7448, (B) *M. hyopneumoniae* J, and (C) *M. flocculare*.

Bar colors represent the different classes of potential PEP determinant, as indicated. CyPE+SuPE, proteins detected in both CyPE and SuPE samples; CyPE, proteins detected only in CyPE samples; SuPE, proteins detected only in SuPE samples. A bar representing the overall number of genes encoding each class of PEP determinants in the corresponding mycoplasma genome was included for reference (EG).

CyPE and SuPE. Interestingly, in *M. flocculare*, the P95 (MFC_00492) and P60-like (MFC_01236) adhesins, in CyPE, and the P97 copy-2 adhesin (MFC_00472), in SuPE, were overrepresented in comparison to *M. hyopneumoniae* 7448.

Proteases are often involved in the virulence of several pathogens, including pathogenic mycoplasmas. In the protease repertoires identified in *M. hyopneumoniae* strains and *M. flocculare* samples, several quantitative differences were observed, which are suggestive of differential

Table 1. Potential virulence-related proteins overrepresented ($p < 0.05$ and FC > 1.5) in *M. hyopneumoniae* 7448 samples. Association with virulence was based on the cited references.

		Fold-changes[1]				
		CyPE[2]		SuPE[2]		
NCBI accession number	Protein name	MHP7448/ MHPJ	MHP7448/ MFC	MHP7448/ MHPJ	MHP7448/ MFC	Reference
MHP7448_0210	ABC transporter ATP-binding protein	2.93	3.13	-	-	[34]
MHP7448_0314	ABC transporter ATP-binding protein	-	2.67	2.35	2.42	[34]
MHP7448_0315	ABC transporter ATP-binding protein	-	-	1.84	-	[34]
MHP7448_0452	ABC transporter ATP-binding protein	-	-	-	3.04	[34]
MHP7448_0129	Aminopeptidase	-	12.75	-	-	[10]
MHP7448_0051	ATP synthase subunit alpha	2.96	-	-	2.28	[70]
MHP7448_0101	ATP-dependent protease binding protein	-	-	-	4.77	[10]
MHP7448_0068	Chaperone protein DnaJ	-	-	-	5.20	[10]
MHP7448_0507	Dihydrolipoyl dehydrogenase	-	-	-	2.61	[41]
MHP7448_0075	Elongation factor G	-	-	-	3.34	[71]
MHP7448_0056	Elongation factor Ts	-	-	-	10.00	[71]
MHP7448_0523	Elongation factor Tu	-	-	-	2.61	[72]
MHP7448_0263	Energy-coupling factor transporter ATP-binding protein EcfA1	-	5.76			[73]
MHP7448_0464	Leucyl aminopeptidase	-	-	2.09	3.95	[10]
MHP7448_0133	Lipase-esterase	-	13.40	-	-	[74]
MHP7448_0137	L-lactate dehydrogenase	-	62.92	-	-	[36]
MHP7448_0524	Lon protease (ATP-dependent protease La)	-	-	-	3.17	[10]
MHP7448_0173	Methionine aminopeptidase	-	3.86	-	-	[10]
MHP7448_0082	NADH oxidase	-	3.90	-	2.48	[75]
MHP7448_0521	Oligoendopeptidase F	-	-	1.59	3.74	[27]
MHP7448_0501	Oligopeptide ABC transporter ATP-binding protein	-	-	-	4.32	[34]
MHP7448_0360	p37-like ABC transporter substrate-binding lipoprotein	-	-	4.24	-	[34]
MHP7448_0272	p97-like protein	2.50	-	-	-	[10]
MHP7448_0161	Phosphopentomutase	3.28	-	-	-	[42]
MHP7448_0656	Prolipoprotein p65	-	4.67	-	2.67	[10]
MHP7448_0376	PTS system ascorbate-specific transporter subunit IIC	-	-	1.58	-	[76]
MHP7448_0375	PTS system enzyme IIB component	3.61	-			[76]
MHP7448_0005	Putative MgpA-like protein	2.84	-	-	-	[10]
MHP7448_0116	Pyruvate dehydrogenase	-	16.00	-	4.35	[41]
MHP7448_0115	Pyruvate dehydrogenase E1-alpha subunit	-	2.61	-	12.91	[41]
MHP7448_0037	Ribonuclease R	3.60	3.03	-	-	[10]
MHP7448_0096	Thiol peroxidase	5.63	-	-	-	[10]
MHP7448_0384	Thioredoxin	-	-	1.55	-	[10]
MHP7448_0098	Thioredoxin reductase	-	-	-	2.81	[10]

[1] Fold changes were based on emPAI quantitative values of MHP7448 divided by those of MHPJ or MFC.
[2] Dashes means that the determined *M. hyopneumoniae* 7448 protein were not differentially abundant in CyPE and/or SuPE and, in comparison to *M. hyopneumoniae* J and/or *M. flocculare*

mechanisms for regulation of protein abundance and subcellular localization. Comparing proteases found in both *M. hyopneumoniae* 7448 and J SuPE, the oligoendopeptidase F and leucyl aminopeptidase were overrepresented in the pathogenic mycoplasma (MHP7448_0521, and MHP7448_0464, respectively). In comparison to *M. flocculare*, 6 proteases were overrepresented in *M. hyopneumoniae* 7448 samples, as follows. An aminopeptidase (MHP7448_0129) and the methionine aminopeptidase (MHP7448_0173) were more abundant in *M. hyopneumoniae* 7448 CyPE. The oligoendopeptidase F (MHP7448_0521), the leucyl aminopeptidase (MHP7448_0464), the ATP-dependent protease binding protein (MHP7448_0101), and the lon protease (MHP7448_0524) were more abundant in *M. hyopneumoniae* 7448 SuPE. Interestingly, the MHP7448_0129 aminopeptidase, overrepresented in *M. hyopneumoniae* 7448 CyPE, was differentially enriched in *M. flocculare*, being ~ 15 times more abundant in SuPE.

Redox balancing proteins can be also associated with virulence of several pathogens, including mycoplasmas, and some of them were differentially represented in the performed proteomic analyzes. *M. hyopneumoniae* neutrophil activating factor (MHP7448_0457) was detected only in *M. hyopneumoniae* 7448 CyPE. Regarding proteins differentially abundant, a thiol peroxidase (MHP7448_0096) and a thioredoxin (MHP7448_0384) (detected in CyPE and SuPE, respectively) were overrepresented in *M. hyopneumoniae* 7448 in comparison to *M. hyopneumoniae* J. In comparison to *M. flocculare*, a NADH oxidase (MHP7448_0082) and a thioredoxin reductase (MHP7448_0098) were overrepresented in the pathogenic mycoplasma.

Membrane transport proteins, such as ABC transporters, permeases and PTS system proteins, correspond to ~ 12% of the proteins encoded by *M. hyopneumoniae* and *M. flocculare* genomes. Around 47% (25) of membrane transporters species detected by LC-MS/MS were

shared among the three analyzed proteomes. Among these membrane transporters, 6 (3 from CyPE and 3 from SuPE) and 3 (from SuPE) proteins were overrepresented and underrepresented, respectively, in *M. hyopneumoniae* 7448 in comparison to *M. hyopneumoniae* J. In comparison to *M. flocculare*, 7 (3 from CyPE and 4 from SuPE) and 1 (from CyPE) membrane transporters were overrepresented and underrepresented, respectively, in *M. hyopneumoniae* 7448.

Besides the canonical virulence-related proteins described above, *M. hyopneumoniae* 7448 also presents several potential virulence-related enzymes that were differentially represented in comparison to *M. hyopneumoniae* J, and *M. flocculare*. In comparison to *M. hyopneum oniae* J, a phosphopentomutase (MHP7448_0161), and a ribonuclease (MHP7448_0037) were 3–4 times more abundant in *M. hyopneumoniae* 7448 CyPE. Comparing to *M. flocculare*, a lipase-esterase (MHP7448_0133), a ribonuclease (MHP7448_0037), two glycolytic enzymes, namely lactate dehydrogenase (LDH, MHP7448_0137), and pyruvate dehydrogenase (represented by three of its four subunits: MHP7448_0116, MHP7448_0115, and MHP7448_0507), a chaperone DnaJ (MHP7448_0068), and three translation elongation factors (MHP7448_0075, MHP7448_0056 and MHP7448_0523) were from 2.6 to ~ 63 times more abundant in *M. hyopneumoniae* 7448.

Overall, these results showed important qualitative and quantitative differences in virulence-related proteins that might be PEP determinants. Importantly, along with these previously described virulence-related proteins, at least 47 other proteins were overrepresented in *M. hyopneumoniae* 7448 proteome in comparison to the samples of non-pathogenic mycoplasmas. The potential of these proteins as PEP determinants deserves further investigation.

Differences between the protein repertoires of M. hyopneumoniae J and M. flocculare

The whole cell proteomes of *M. hyopneumoniae* J and *M. flocculare* were also qualitative and quantitatively analyzed and compared between each other. Among the proteins detected in *M. hyopneumoniae* J and *M. flocculare* samples, 26 and 41 proteins were exclusively detected, respectively (see Figure 1). Among the proteins shared between *M. hyopneumoniae* J and *M. flocculare*, 21 and 68 proteins from CyPE and SuPE, respectively, were differentially abundant (Supplementary Table 5C). The differences in abundance of both CyPE and SuPE proteins ranged from ~ 1.6 to ~ 19-times fold. Regarding CyPE differentially abundant proteins, 4 and 17 were overrepresented in *M. hyopneumoniae* J and *M. flocculare*, respectively. On the other hand, 63 and 5 SuPE proteins were overrepresented in *M. hyopneumoniae* J and *M. flocculare*, respectively. All 4 *M. hyopneumoniae* J overrepresented CyPE proteins were also overrepresented in SuPE samples. Interestingly, two *M. hyopneumoniae* J proteins, an ABC transporter (MHJ_0450) and an arginine-tRNA ligase (MHJ_0012), were differentially enriched, once they were underrepresented in CyPE and overrepresented in SuPE.

Overall, the comparisons between the proteomes of *M. hyopneumoniae* J and *M. flocculare* did not provide evidence of common features that could be clearly associated with the lack of virulence of these related bacteria. However, the observed qualitative and quantitative differences point out to physiological differences between them that deserve further investigation.

Functional enrichment analyzes of the whole cell protein sets of M. hyopneumoniae 7448 and J, and M. flocculare

GO functional enrichment analyzes were performed for the whole cell protein sets of all mycoplasma samples to provide clues on functional differences between strains and species. Totals of 292 *M. hyopneumoniae* 7448 proteins (Supplementary Table 6A), 292 *M. hyopneumoniae* J proteins (Supplementary Table 6B), and 286 *M. flocculare* proteins (Supplementary Table 6C) were categorized according to GO terms into "biological process" (BP), "molecular function" (MF), and "cellular component"(CC) categories. No annotations were retrieved for 52, 51, and 29 proteins of *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare*, respectively. Several functional BP, CC, and MF subcategories were commonly overrepresented in all mycoplasma samples. On the other hand, some functional subcategories were exclusively found as overrepresented in each of the analyzed samples, as follows. "Cellular macromolecule metabolic process", "phosphorus metabolic process" and "ribose phosphate metabolic process" (BP subcategories); and "nucleic acid binding", "oxidoreductase activity", and "translation factor activity, RNA binding" (MF subcategories) were enriched only in *M. hyopneumoniae* 7448. For *M. hyopneumoniae* J. only the MF subcategories "hydrolase activity", "nucleoside-triphosphatase activity", and "pyrophosphatase activity" (MF) were exclusively enriched. Finally, some subcategories involved in nucleotide metabolism, as "pyridine nucleotide metabolic process", "nucleobase-containing compound biosynthetic process", "aromatic compound biosynthetic process" (BP subcategories), and other nucleotide-metabolism related MF subcategories were exclusively enriched in *M. flocculare*.

The performed GO functional analyzes showed that *M. hyopneumoniae* 7448 and J strains and *M. flocculare* present overall metabolic similarities, as expected. However, some interesting differences were highlighted among them, pointing out specific functional distinctions with possible impact for their proliferation, and survival capacities in the natural host.

Uncharacterized proteins detected in the M. hyopneumoniae 7448 and J, and M. flocculare proteomes

Our proteomic data provided experimental validation for 70, 73 and 69 genes previously regarded as hypothetical for *M. hyopneumoniae* 7448 and J, and *M. flocculare*, respectively, establishing interesting subsets of mycoplasma uncharacterized proteins (Supplementary Table 3), which deserve further attention. The Venn diagram in Figure 4(A), summarizes the exclusive and shared repertoires of detected *M. hyopneumoniae* 7448 and J, and *M. flocculare* uncharacterized proteins. Cell fraction analyzes revealed that ~ 44% of the uncharacterized protein species were found in both CyPE and SuPE (Figure 4(B)). A large number of uncharacterized protein species were exclusively detected in SuPE samples from *M. hyopneumoniae* 7448 (25, 35%) and J (39, 53%). For *M. flocculare*, only 24% of uncharacterized proteins were detected in SuPE.

In silico functional predictions were performed in order to provide clues on the functional roles of the detected uncharacterized proteins. A total of 41 different domains from the Pfam database were found distributed among 52 out of the total of 109 different uncharacterized proteins species detected in the analyzed samples (Supplementary Table 7). The "N-6 DNA methylase" domain was exclusively found in the *M. hyopneumoniae* 7448 set of uncharacterized proteins, while the DUF1410, 'DUF4231' and "tRNA synthetases class II" domains were exclusively found in the *M. hyopneumoniae* J set. Finally, domains related to replication initiation, peptidase and recombinase functions were found exclusively in the *M. flocculare* set.

Among the uncharacterized proteins shared between *M. hyopneumoniae* 7448 and J, and/or between *M. hyopneumoniae* 7448 and *M. flocculare*, 14 virulence-related domains were identified, including domains of potential peptidases, lipases, nucleases, permeases, thioredoxins and chaperones, were found distributed among 18 protein species. Among these proteins, only 4 were overrepresented in *M. hyopneumoniae* 7448, namely MHP7448_0431, bearing a "phosphatidylethanolamine-binding" domain; MHP7448_0064, bearing a "AAA domain"; MHP7448_0522, bearing a "GDSL-like lipase/acylhydrolase" domain; and MHP7448_0148, bearing a 'Hsp33' domain. While MHP7448_0431 and MHP7448_0064 were overrepresented in comparison to *M. hyopneumoniae* J, MHP7448_0431, MHP7448_0522 and MHP7448_0148 were overrepresented in comparison to *M. flocculare*.

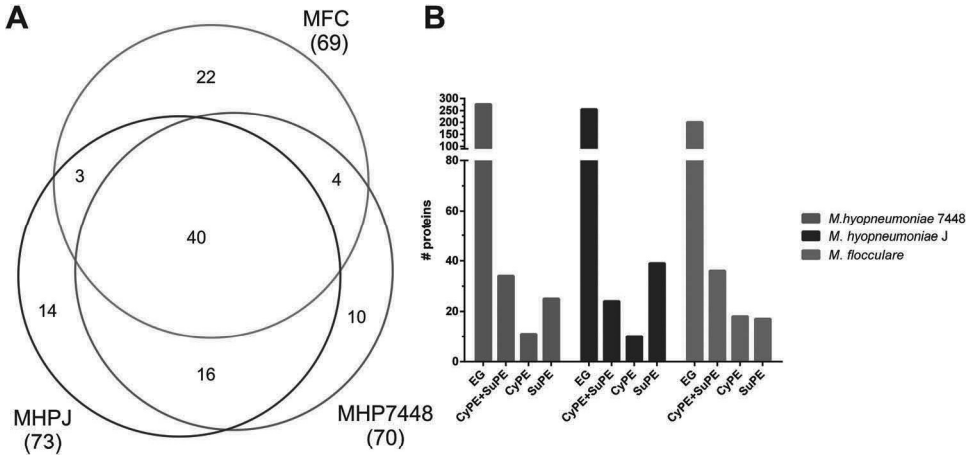

Figure 4. Overview of the uncharacterized proteins identified in *M. hyopneumoniae* 7448 and J, and *M. flocculare* samples. (A) Venn diagram of uncharacterized proteins detected in *M. hyopneumoniae* 7448 (MHP7448) and J (MHPJ), and *M. flocculare* (MFC) samples. Overall numbers of proteins identified for each sample between parentheses. The numbers of uncharacterized proteins exclusively detected in each sample or shared between them are indicated within the Venn diagram. (B) Distribution of uncharacterized proteins detected in CyPE and/or SuPE samples. Bar colors represent the different analyzed mycoplasma samples. CyPE+SuPE, proteins detected in both CyPE and SuPE samples; CyPE, proteins detected only in CyPE samples; SuPE, proteins detected only in SuPE samples. A bar representing the overall number of genes encoding uncharacterized proteins in the corresponding mycoplasma genome was included for reference (EG).

Discussion

Bacterial pathogenicity and virulence are multifactorial features that can be better assessed in comparative studies at the protein level, as protein abundance is the result of transcriptional regulation, post-translational processing and/or protein degradation. In this study, we compared the protein repertoires of cytoplasmic and surface-enriched protein fractions, comprehending the whole-cell proteomes, from the pathogenic and non-pathogenic *M. hyopneumoniae* strains (7448 and J, respectively), and *M. flocculare*, a non-pathogenic related species. For the first time, subcellular fractions of *M. hyopneumoniae* and *M. flocculare* were comparatively assessed using high-sensitivity high-resolution mass spectrometry. Qualitative and quantitative differences between the pathogenic *M. hyopneumoniae* 7448 and its non-pathogenic were found, involving potential PEP determinants, such as adhesins, proteases, and proteins related to redox balancing or membrane trafficking.

Cell fractioning procedures are useful to reduce proteome complexity, allowing the enrichment of low-abundance proteins. They improve the efficiency of MS-based protein identification and allow the association of different sets of proteins to specific cell compartments [17]. The carried out fractioning approach allowed to generate soluble fractions, enriched with cytoplasmic proteins (CyPE), and insoluble fractions, enriched with surface proteins (SuPE). For SuPE preparation, protein solubilization was carried out using the RapiGest SF surfactant, instead of the usual Triton X-114 or SDS solubilization protocols [18]. This surfactant allowed efficient protein solubilization in a one-step procedure and improved MS-protein identification. *In silico* subcellular localization prediction associated with the quantitative proteomics of CyPE and SuPE, confirmed their enrichment with cytoplasmic and surface proteins, respectively, for all analyzed mycoplasma samples. A previous *M. hyopneumoniae* 7448 surface protein survey carried out by our group identified only 34 surface-predicted proteins detected using a biotin cell surface labeling approach [19]. Our fractionation/solubilization approach, in turn, allowed the identification of 111 surface-predicted proteins in the *M. hyopneumoniae* 7448 SuPE (38% of the predicted surfaceome).

The cell fractioning approach combined with a high-resolution and sensitivity LC-MS/MS provided a high proteome coverage for all three mycoplasmas analyzed. The LC-MS/MS approach sensitivity was evidenced by comparing our data to those published by Pinto *et al.* (2009). In comparison to the former data, proteome coverage was improved 28% (from 22% to 50%) for *M. hyopneumoniae* 7448, and 27% (from 24% to 51%) for *M. hyopneumoniae* J. The remaining ~ 50% of predicted proteins not covered by our proteomic data may not have been detected due to their low abundance or lack of expression in culture conditions.

M. hyopneumoniae 7448 shared ~ 70% of the detected proteins with *M. hyopneumoniae* J and *M. flocculare*. Despite these high similarities between the sets of proteins detected for *M. hyopneumoniae* strains and *M. flocculare*, many qualitative and quantitative differences were detected, several of them likely associated with pathogenicity/PEP determination. Within the sets of proteins differentially represented in *M. hyopneumoniae* 7448 in comparison to the non-pathogenic samples, there are representatives of several classes of proteins and/or functions that may be potential PEP determinants, such as adhesins, proteases, oxidative stress-related proteins, and membrane transporters, among others.

Genomic comparative analyzes demonstrated that the sets of adhesin-encoding genes from *M. hyopneumoniae* 7448 and J, and *M. flocculare* are quite similar, containing few qualitative differences between the adhesin repertoires of *M. hyopneumoniae* and *M. flocculare* [9]. The only differences are the absence of *M. flocculare* orthologs for one P97 paralog (P97 copy-1, MHP7448_0198), and one P102 paralog (P102 copy-1, MHP7448_0199), and some rearrangements in *M. hyopneumoniae* genomic regions containing adhesin genes in comparison to *M. flocculare*. Despite these differences, the overall high qualitative similarity between the *M. hyopneumoniae* and *M. flocculare* adhesin sets was confirmed at proteomic level by the data described here. However, our data also pointed out some interesting quantitative differences, as three adhesins (P97-like, MgpA-like and P65) were more abundant in *M. hyopneumoniae* 7448 than in the non-pathogenic mycoplasmas, which may be associated with the higher adherence capacity of pathogenic *M. hyopneumoniae*. Conversely, four adhesins were more abundant in the non-pathogenic mycoplasmas. P95 and P97, for example, were more abundant in *M. flocculare* than in *M. hyopneumoniae* 7448. However, the *M. flocculare* orthologs are quite divergent (only ~ 55% of sequence identity to the *M. hyopneumoniae* orthologs), which may imply different adhesion properties. Moreover, *M. flocculare* has only one copy of P97 (MFC_00472), while *M. hyopneumoniae* has two, and, in this case, the overrepresentation of the single *M. flocculare* P97 may be resultant of a compensating mechanism.

Additionally, we observed higher peptide coverages in CyPE in comparison to those in SuPE, for *M. hyopneumoniae* and *M. flocculare* proteins, including adhesins. This suggests that these proteins are more fragmented in the cell surface than in in the cytoplasm, when they are expected to be mostly unprocessed. Previous studies have showed that adhesins are targets of post-translational proteolytic events [20–24] which can be differential between *M. hyopneumoniae* strains [15]. Along with differential adhesin abundance, the possibly differential adhesin post-translational proteolytic processing likely impact on bacterial pathogenicity and deserve further investigation.

As mediators of post-translational proteolytic events and other important cell processes, proteases play an important role to shape the *M. hyopneumoniae* proteome. Most of the proteases found in the whole cell proteomes of *M. hyopneumoniae* strains and *M. flocculare* were detected in both CyPE and SuPE. Interestingly, most of the overrepresented proteases of *M. hyopneumoniae* 7448 in comparison to *M. hyopneumoniae* J and *M. flocculare* were detected in SuPE. These differences in protease abundance between subcellular fractions and between pathogenic and non-pathogenic mycoplasmas could be resultant of differential enzyme activity or regulation for their targeting to preferential substrates in cell surface. With that, specific proteolytic activities could be targeted, for example, to the processing of surface adhesins.

Some of the proteases overrepresented in *M. hyopneumoniae* had their activities experimentally assessed [25–27]. Interestingly, *M. hyopneumoniae* leucyl aminopeptidase has been associated with plasminogen, heparin and foreign DNA binding and is localized on mycoplasma cell surface [26], which corroborated its higher abundance in *M. hyopneumoniae* 7448 SuPE. Moreover, oligoendopeptidase F and XAA-PRO aminopeptidase were previously associated with host kallikrein-kinin system, participating in inflammatory processes [27]. Overall, overrepresentation of proteases in the surface of *M. hyopneumoniae* 7448, along with previous functional studies, indicate the involvement of these enzymes with important pathogenicity-related mechanisms from adhesion to host immunomodulation.

Endogenous production of hydrogen peroxide through glycerol metabolism is essential for cytotoxicity of pathogenic mycoplasmas, as *Mycoplasma pneumoniae* and *Mycoplasma mycoides* subsp. *mycoides* [28,29]. In line with that, it was recently demonstrated that pathogenic strains of *M. hyopneumoniae* were able to produce hydrogen peroxide from glycerol metabolism, but that the non-pathogenic strain J and *M. flocculare* were not [11]. *M. hyopneumoniae* uptakes and metabolizes glycerol, while *M. flocculare* does not, failing to produce cytotoxic levels of hydrogen peroxide, which can be explained by the absence, in the *M. flocculare* genome, of the *glpO* gene, related to glycerol metabolism and hydrogen peroxide production [12].

Among proteins involved with oxidoreduction processes, a neutrophil activating factor was exclusively detected in *M. hyopneumoniae* 7448 CyPE. In *Helicobacter pylori*, this protein was previously related to neutrophil activation by the production of reactive oxygen species (ROS) [30]. Moreover, several redox balancing proteins were more abundant in *M. hyopneumoniae* 7448 than in *M. hyopneumoniae* J and *M. flocculare*. These results agreed with the functional enrichment analyzes, which demonstrated that the "oxidoreductase activity" subcategory, including all detected proteins related to redox balancing, was exclusively enriched in *M. hyopneumoniae* 7448. These differentially abundant proteins can be considered potential PEP determinants, due to their importance for bacterial survival in the context of endogenous (mycoplasma) and exogenous (host) ROS production [31–33]. For *M. flocculare*, its inability to produce endogenous hydrogen peroxide may be associated with its commensal nature, being less harmful to the host.

Membrane transporters have been described as virulence-related proteins, as they may be associated with multidrug resistance, metal ions uptake, and cell attachment [34], which are important for bacterial survival, and host colonization. *M. hyopneumoniae* and *M. flocculare* genomes have ~ 80 membrane transporters coding genes each, including genes coding for ABC transporters, permeases and PTS systems. In the LC-MS/MS analyzes, ~ 68% of the sets of membrane transporters species identified in *M. hyopneumoniae* and *M. flocculare* predicted proteomes were detected, with a partial (~ 50%) overlapping. Moreover, abundance differences were found between membrane transporters orthologs shared by *M. hyopneumoniae* 7448 and *M. hyopneumoniae* J, or by *M. hyopneumoniae* 7448 and *M. flocculare*. Overall, these evident qualitative and quantitative differences among the sets of membrane transporters of *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare* are suggestive of substantial differences in transporting activities/capabilities and may also contribute to their differential virulence/pathogenicity.

Many proteins not classically related to virulence were also differential represented between *M. hyopneumoniae* 7448 and its non-pathogenic counterparts analyzed here. Functional enrichment analyzes showed some important metabolic subcategories specifically

enriched in the *M. hyopneumoniae* 7448 whole cell proteome. The "phosphorous metabolic process" subcategory, which includes several glycolytic enzymes and kinases, and the "RNA binding" subcategory, which includes ribosomal proteins, translational elongation factors and aminoacyl tRNA ligases, were exclusively enriched in this pathogenic mycoplasma. In agreement to the functional enrichment analyzes, several proteins with canonical functions in metabolic pathways were overrepresented in *M. hyopneumoniae* 7448 protein repertoire, including the glycolytic enzymes LDH and pyruvate dehydrogenase, the pentose pathway enzyme phosphopentomutase, and translation-related proteins. Overall, the exclusive enrichment of all these metabolic functions suggests a higher metabolic capacity for the pathogenic *M. hyopneumoniae* strain, which may favor its proliferation and survival, contributing to the colonization, and infection of the porcine respiratory tract.

Glycolytic enzymes and other differential *M. hyopneumoniae* 7448 proteins not usually regarded as virulence factors, such as proteins involved in pentose phosphate pathway, DNA replication, and translation may have also alternative (moonlighting) functions of relevance for pathogenicity [35–37]. For instance, LDH is highly immunogenic and may have an immunomodulatory role [38,39], while pyruvate dehydrogenase and phosphopentomutase are proteins that play roles in adherence to the host extracellular matrix and DNA repair, respectively [40–42].

Around 37% of the sequenced genomes of *M. hyopneumoniae* strains and *M. flocculare* codes for hypothetical proteins. For pathogenic species, such set of hypothetical proteins is of particular interest, once it represents a potential reservoir of unknown virulence factors. For *M. pneumoniae*, many novel virulence factors were predicted upon *in silico* analyzes of hypothetical proteins [43]. In our study, several *M. hyopneumoniae* and *M. flocculare* coding DNA sequences (CDSs) whose putative products have been annotated as "hypothetical proteins" had their proteins products experimentally detected by LC-MS/MS. This allowed to confirm these CDSs as functional genes, and to change the status of their products to that of "uncharacterized proteins". Among the detected uncharacterized proteins, several functional domains were predicted, including virulence-related ones, and most of them were conserved among the orthologs. More importantly, abundance differences between *M. hyopneumoniae* 7448 and its assessed non-pathogenic counterparts were observed for some of the uncharacterized proteins bearing functional domains, including virulence-related ones. Future analyzes of these and other uncharacterized proteins along with the characterization of their functional domains will be important steps towards the elucidation of their functions in *M. hyopneumoniae* biology and their possible roles as novel virulence factors.

Conclusion

Our results provided a comprehensive profiling of the whole cell proteomes of two *M. hyopneumoniae* strains and *M. flocculare*, and an extended list of tens of candidates to pathogenicity determinants, beyond those classically described. Several protein classes with potential virulence-related functions were identified as overrepresented in the *M. hyopneumoniae* 7448 pathogenic strain, including adhesins, proteases, oxidative stress proteins, membrane transporters, and proteins with moonlighting functions, along with many so far uncharacterized proteins. Based on our proteomics results, the pathogenic nature of *M. hyopneumoniae* may be explained, at least in part, by the overrepresentation of several virulence-related proteins. These overrepresented proteins are involved in a wide range of biological processes, including adhesin processing and cell adhesion regulation, detoxification, overall metabolism regulation, and host-pathogen cell trafficking, among others. Although no specific commensalism determinants were found, the underrepresentation of several virulence-related proteins encoded by the non-pathogenic mycoplasmas may be a key point to explain their commensal natures.

Several of the identified proteins in *M. hyopneumoniae* strains and *M. flocculare* repertoires deserve future studies to elucidate mechanisms related to pathogenicity or commensalism, respectively. Of particular interest will be proteins with unknown function or with possible moonlighting functions overrepresented in the pathogenic *M. hyopneumoniae* 7448 strain. Moreover, the identification and characterization of *M. hyopneumoniae* virulence factors is of upmost relevance to discover new targets for the development of novel diagnostic methods, therapeutic drugs, and preventive vaccines against PEP.

Materials and methods

Bacterial growth conditions

M. hyopneumoniae pathogenic strain 7448 was isolated from an infected swine from Lindóia do Sul (SC, Brazil) [7]. *M. hyopneumoniae* non-pathogenic strain J (ATCC 25,934), and the non-pathogenic *M. flocculare* (ATCC 27,716) were acquired from American Type Culture Collection by the Empresa Brasileira de Pesquisa

Agropecuária-Centro Nacional de Pesquisa de Suínos e Aves (EMBRAPA-CNPSA, Concórdia, SC, Brazil). For soluble and insoluble protein extracts, respectively, all bacteria were cultivated in 50 mL and 100 mL of Friis medium [44] for 48 h [45], at 37°C. Cultures were carried out independently in triplicates (biological replicates), and immediately used for protein extraction.

Protein extraction and sample preparation for mass spectrometry

For protein extraction, cultured mycoplasma cells were pelleted by centrifugation (3500 x *g*, 15 min, 4°C), and washed three-times with PBS (pH 7.4). Cells were resuspended and lysed by sonication at 25 Hz in an ice bath by five 30 s cycles with 1 min intervals between pulses. The lysates were centrifuged at 10,000 x *g*, for 20 min, at 4°C and the supernatant (soluble fraction) was recovered for proteomics analyzes. The pellet (insoluble fraction) was resuspended in RapiGest SF Surfactant (Waters Corporation, Number 186,001,861). Soluble and insoluble protein extracts were quantified using the microBCA Protein Assay Kit (Thermo Fischer Scientific, Number 23,235) using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific). Three protein extracts were independently produced to provide the three biological replicates for each sample.

Samples containing 100 µg and 50 µg of proteins from the soluble and insoluble fractions, respectively, were treated for MS analyzes. For soluble fraction analyzes, proteins were precipitated with TCA 20%-acetone, incubated for 16 h at 4°C, and further centrifuged at 20,000 × g for 10 min. Protein pellets were dried and then solubilized with 8 M urea. Next, proteins were reduced with 2 µg of DTT (Bio-Rad, Number 161–0611) at 37°C for 1 h, and alkylated with 10 µg of iodoacetamide (Bio-Rad, Number 163–2109) in the dark, at room temperature. Protein samples were diluted to a final 1 M urea concentration, and further digested with 1 µg of trypsin (Promega, Number V5280). For insoluble fraction analysis, samples resuspended in RapiGest SF were reduced with DTT (Bio-Rad) at 60°C for 30 min to a final concentration of 5 mM and alkylated with iodoacetamide (Bio-Rad) 15 mM (final concentration) at room temperature for 30 min in the dark. Proteins were then digested overnight with 0.5 µg of trypsin (Promega) at 37°C, and RapiGest SF was removed as recommended by the manufacturer (Waters). Resulting soluble and insoluble fractions peptides were desalted in HLB cartridges (Waters, Number 186,000,383), and eluted with 50% acetonitrile/0.1% TFA. Peptides were then lyophilized using a Concentrator Plus (Eppendorf), prior to MS analyzes.

Mass spectrometry analyzes

Processed peptide samples were analyzed for protein identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described [16,46]. Briefly, each peptide sample was reconstituted using 0.1% formic acid in water, loaded onto a nanoAcquity HPLC system (Waters Corporation, MA, USA). A two-step LC was performed, using first a trap column PepMap 100 C18 LC column (300 µm x 5 mm) (Thermo Fischer Scientific, IL, USA), at a flow rate of 5 µl/min, and then an Easy-Spray Column PepMap RSLC C18 (75 µm x 15 cm) analytical column (Thermo Fischer Scientific). For the gradient elution, the mobile phase solvents consisted of 0.1% formic acid in water (solvent A), and 0.1% formic acid in acetonitrile (Burdick and Jackson) (solvent B). The gradient flow was set at 0.3 µl/min. The elution profile consisted of a hold at 5% solvent B for 5 min, followed by a ramp up to 35% solvent B over 25 min; a ramp up to 95% solvent B in 5 min; and a hold at 95% for 5 min, prior to a return to 5% solvent B in 5 min, and re-equilibration at 5% solvent B for 20 min. After LC, the peptides were introduced into a MS/MS Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer (Thermo Fischer Scientific). A 2.0 kV voltage was applied to the nano-LC column. The mass spectrometer was programmed to perform data-dependent acquisition by scanning the mass-to-charge (m/z) range from 400 to 1600, at a nominal resolution setting of 60,000 for parent ion acquisition. For the MS/MS analyzes, the mass spectrometer was programmed to select the top 15 most intense ions with two or more charges. Each biological replicate was independently analyzed by LC-MS/MS two times (technical replicates).

LC-MS/MS data analyzes

The MS/MS raw data were processed using msConvert version 3 (ProteoWizard) [47], and the peak lists were exported in the Mascot Generic Format (.mgf). MS/MS processed data were analyzed using Mascot Search Engine version 2.3.02 (Matrix Science, MA, USA) against local databases available for *M. hyopneumoniae* 7448 and J strains, and *M. flocculare*. These local databases were derived from the fully sequenced genomes from *M. hyopneumoniae* 7448 (920,079 bp), *M. hyopneumoniae* J (897,405 bp), and *M. flocculare* (763,948 bp), and included all deduced amino acid sequences (695, 672, and 581,

respectively) from the corresponding genomes annotation (Siqueira et al 2013; Vasconcelos et al 2005), available at NCBI (https://www.ncbi.nlm.nih.gov/protein/) and Uniprot (http://www.uniprot.org/). The MASCOT search parameters for protein identification included a fragment ion mass tolerance of 0.5 Da, peptide ion tolerance of 7 ppm, and three missed cleavages of trypsin. Carbamidomethylation of cysteine was specified as a fixed modification, whereas the oxidation of methionine, acetylation of lysine and N-terminal ends of proteins, and phosphorylation of tyrosine and serine/threonine were specified as variable modifications [48].

Scaffold software version 4.8.1 (Proteome Software Inc., OR, USA) was used to validate the peptide and protein identifications. The peptide identifications were accepted if they could be established at greater than 99.0% probability as assigned by the Peptide Prophet algorithm [49]. The protein identifications were accepted if they could be established at greater than 95% probability as assigned by the Protein Prophet algorithm [50]; were based on at least 2 identified peptides; and were detected in at least two out of three replicates (both biological and technical).

Identification of ortholog proteins among M. hyopneumoniae 7448 and J, and M. flocculare

In order to allow comparisons among protein repertoires from *M. hyopneumoniae* 7448 and J, and *M. flocculare*, ortholog sequences were determined using OrthoFinder [51]. Orthologs were then established based on the resulting bidirectional best hits, using as parameters identity ≥ 40% and a cutoff value of $1e^{-6}$.

In silico subcellular localization predictions

Proteins identified in soluble and insoluble fractions of *M. hyopneumoniae* 7448 and J strains, and *M. flocculare* were analyzed *in silico* to predict their subcellular localization, being classified as surface or cytoplasmic proteins. Membrane proteins were initially predicted based on positive predictions as lipoproteins, using LipoP 1.0 [52], and PRED-LIPO [53]. Non-lipoproteins were then analyzed for transmembrane domain prediction using TMHMM v.2.0 [54], Phobius [55], HMMTOP [56], CW-PRED [57], and HMM-TM [58]. Non-transmembrane proteins were further analyzed for subcellular localization using PSORTb v. 3.0.2 [59], iLoc-Gpos [60], and CELLO v.2.5 [61].

Proteins not predicted as membrane proteins were then classified as secreted or cytoplasmic. Secreted proteins were predicted based on the presence of signal peptide or on non-classical secretion prediction. Signal peptide predictions were made using SignalP 4.1 [62], Phobius [55], and PrediSi [63]. Non-classical secretion was predicted using SecretomeP 1.0 [64]. Remaining proteins, not classified as membrane or secreted proteins, were considered cytoplasmic proteins. For any given prediction, coincidence in all or at least most of the used predictors was required for validation.

Quantitative and qualitative comparisons between LC-MS/MS data of insoluble and soluble protein extracts from M. hyopneumoniae 7448 and J, and M. flocculare

To confirm the enrichment of surface proteins in the insoluble fractions, the LC-MS/MS datasets of proteins identified in the *M. hyopneumoniae* 7448 and J, and *M. flocculare* insoluble fractions were compared to those of the corresponding soluble fractions. For that, differentially represented proteins, exclusively detected or more abundant in the insoluble protein fraction in comparison to the soluble extract of the same species or strain, were analyzed based on subcellular localization predictions. Protein abundance was measured based on normalized spectral abundance factor (NSAF) values [65] and quantitative differences between proteins detected in both insoluble and soluble protein fractions were statistically analyzed in Scaffold software using the Student's *t*-test, with the Benjamini-Hochberg FDR multiple-testing correction. A p-value < 0.05 was considered statistically significant. Proteins with differential abundances between surface-enriched and soluble protein extracts were represented in heat-maps using the Heatmapper web server (http://www.heatmapper.ca) using the Z-score calculation of NSAF values.

Comparative quantitative analyzes of proteins shared between M. hyopneumoniae 7448, J, and M. flocculare

For quantitative comparisons between ortholog proteins shared between (i) *M. hyopneumoniae* 7448 and *M. hyopneumoniae* J; (ii) *M. hyopneumoniae* 7448 and *M. flocculare*; and (iii) *M. hyopneumoniae* J and *M. flocculare*, the analyzes were based on the exponentially modified protein abundance index (emPAI) values [66]. EmPAI values were calculated for each protein in the Scaffold software, not using the normalization option, to allow intraprotein (between ortholog proteins), and intersample (*M. hyopneumoniae* 7448 vs. *M. hyopneumoniae* J; *M. hyopneumoniae* 7448 vs. *M. flocculare*; or *M. hyopneumoniae* J vs. *M. flocculare*) comparisons. The emPAI values were statistically compared using Student's *t*-test using Prism

GraphPad Software version 6 (GraphPad Software, Inc, CA, USA). Fold-changes (FC) were calculated for each pair of ortholog proteins. Proteins with a p-value < 0.05 and a FC > 1.5 were considered differentially abundant by both statistical and FC parameters.

In silico functional analyzes

In silico functional analyzes of *M. hyopneumoniae* and *M. flocculare* proteins identified by LC-MS/MS were based on gene ontology (GO). Mycoplasma identified proteins were submitted to hierarchical GO overrepresentation tests using the Cytoscape 2.6.3 26 plugin BiNGO 2.3 [67]. Custom *M. hyopneumoniae* 7448 and J GO annotation files were acquired from Uniprot (http://www.uniprot.org/). *M. flocculare* GO annotations were acquired using BLAST2GO version 3.0 [68]. For that, online BlastP searches were performed against the NCBInr database and GO mapping, and annotation were performed based on BlastP results (E-value ≤ 1.0×10^{-3}). The ontology files were retrieved from the GO database (http://www.geneontology.org/). Both annotation and ontology files were edited in-house as BiNGO input files. The hypergeometric overrepresentation tests were performed at a 0.05 level of significance, with the Benjamini-Hochberg FDR multiple-testing correction. Uncharacterized proteins were further analyzed in order to predict functional domains using the Pfam software version 29.0 (http://pfam.xfam.org/) [69].

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Disclosure statement

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4.2 Processamento proteolítico diferencial de proteínas de superfície relacionadas à adesão em *M. hyopneumoniae* e *M. flocculare*

O artigo que constitui esta seção, intitulado *Evidences of differential endoproteolytic processing on the surfaces of Mycoplasma hyopneumoniae and Mycoplasma flocculare,* foi publicado na revista Microbial Pathogenesis (https://doi.org/10.1016/j.micpath.2019.103958). Os resultados estão associados aos objetivos 3.2.4 e 3.2.5 desta tese. O artigo possui a primeira autoria compartilhada entre a autora Lais Del Prá Netto Machado e a Dra. Jéssica Andrade Paes. As contribuições dos coautores estão descritas abaixo. O material suplementar (*Supplementary data*) associado está disponível pelo link https://www.sciencedirect.com/science/article/pii/S0882401019318108#appsec1.

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Evidences of differential endoproteolytic processing on the surfaces of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*

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ABSTRACT

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* are genetic similar bacteria that colonize the swine respiratory tract. However, while *M. hyopneumoniae* is a pathogen that causes porcine enzootic pneumonia, *M. flocculare* is a commensal. Adhesion to the respiratory epithelium is mediated by surface-displayed adhesins, and at least some *M. hyopneumoniae* adhesins are post-translational proteolytically processed, producing differential proteoforms with differential adhesion properties. Based on LC-MS/MS data, we assessed differential proteolytic processing among orthologs of the five most abundant adhesins (p97 and p216) or adhesion-related surface proteins (DnaK, p46, and ABC transporter xylose-binding lipoprotein) from *M. hyopneumoniae* strains 7448 (pathogenic) and J (non-pathogenic), and *M. flocculare*. Both surface and cytoplasmic non-tryptic cleavage events were mapped and compared, and antigenicity predictions were performed for the resulting proteoforms. It was demonstrated that not only *bona fide* adhesins, but also adhesion-related proteins undergo proteolytical processing. Moreover, most of the detected cleavage events were differential among *M. hyopneumoniae* strains and *M. flocculare*, and also between cell surface and cytoplasm. Overall, our data provided evidences of a complex scenario of multiple antigenic proteoforms of adhesion-related proteins, that is differential among *M. hyopneumoniae* strains and *M. flocculare*, altering the surface architecture and likely contributing to virulence and pathogenicity.

1. Introduction

Mycoplasma hyopneumoniae is the main etiological agent of porcine enzootic pneumonia (PEP), a chronic respiratory disease that causes major economic losses to the pig industry worldwide [1,2]. It is highly transmissible, as infected swine can be asymptomatic carriers and are capable to infect other susceptible animals. PEP pathogenesis is not fully understood, but it is known that interactions between *M. hyopneumoniae* surface proteins and host ciliated epithelium are essential for disease development [1,3]. *M. hyopneumoniae* has the ability to adhere to the porcine respiratory ciliated cells and induce damage to the cilia, increasing the susceptibility to secondary infections [3–5].

Another closely related mycoplasma species, *Mycoplasma flocculare*, also colonizes the swine respiratory tract [6]. However, it is considered non-pathogenic, once clinical signs, as typical PEP lung lesions and/or obvious ciliary damage, have not been associated to the presence of this mycoplasma. It was shown that *M. flocculare* also adheres to the ciliated cells of the respiratory epithelium, but less than *M. hyopneumoniae* [4].

Colonization of swine respiratory tract by *M. hyopneumoniae* and *M. flocculare* depends on the adhesion to the ciliated epithelial cells, which is mediated mainly by specialized adhesion proteins called adhesins [1]. In this context, the different adhesion capacities of *M. hyopneumoniae* and *M. flocculare* suggested that they have different sets of adhesins. However, comparative genomic and transcriptomic studies have demonstrated that *M. hyopneumoniae* and *M. flocculare* share most of the genes and gene products related to pathogenicity, including most of the adhesin or adhesion-related genes [7,8]. Moreover, comparative proteomic analyses showed few quali-quantitative differences in the

Abbreviations: PEP, porcine enzootic pneumonia; CyPE, cytoplasmic-enriched protein extracts; SuPE, surface-enriched protein extracts; ATXBL, ABC transporter xylose-binding lipoprotein

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adhesin or adhesion-related protein sets among *M. hyopneumoniae* pathogenic (7448) and non-pathogenic (J) strains, and *M. flocculare* [9]. Overall, these findings suggest that there are no major differences in the adhesin repertoires between *M. hyopneumoniae* strains and *M. flocculare* due to gene expression regulation at the transcriptional and translational levels.

On the other hand, several studies have been demonstrated that at least some adhesins presented in *M. hyopneumoniae* cell surface are post-translationally proteolytically processed, generating different adhesin proteoforms with potential differential adhesion properties [10–19]. However, the extant of this adhesin processing in terms of the numbers of targeted proteins, and generated proteoforms remains elusive. So far, proteolytic processing has been assessed for several adhesins and adhesion-related proteins, and for some other surface proteins, as well [10–23]. These studies have been carried out mostly in the *M. hyopneumoniae* 232 (pathogenic) and J (non-pathogenic) strains, but not in comparative approaches. The occurrence of proteolytic processing in additional *M. hyopneumoniae* pathogenic strains or *M. flocculare* adhesins and adhesion-related proteins remains to be investigated.

Here, based on previously LC-MS/MS results [9], the five most abundant adhesins and adhesion-related proteins from *M. hyopneumoniae* 7448 and their respective orthologs from *M. hyopneumoniae* J and *M. flocculare* were comparatively analyzed in order to identify differential proteolytic processing events. Several endoproteolytic cleavage events were mapped in cytoplasm and surface-exposed adhesins and adhesion-related proteins, producing differential antigenic proteoforms among mycoplasma strains or species, and between subcellular compartments. These findings are helpful for the identification of differential *M. hyopneumoniae* 7448 surface-exposed and antigenic adhesin proteoforms, which may be associated to virulence and pathogenicity and point out to new targets for vaccines and/or diagnostic tests development.

2. Materials and methods

2.1. M. hyopneumoniae adhesion-related proteins and associated mass spectrometry data

To define the set of *M. hyopneumoniae* adhesion-related proteins, all proteins previously described as adhesins or related to adhesion based on experimental assays, on binding properties to host extracellular matrix molecules or to the porcine cilia, or on orthology with different mycoplasma species were considered. The defined ortholog sets of adhesion-related proteins for *M. hyopneumoniae* 7448 and J, and for *M. flocculare* are presented in Table S1. All proteins defined as adhesion-related were previously detected and quantified in LC-MS/MS analyses [9], and information regarding protein abundance, differential protein coverages in cytoplasmic and surface-enriched protein extracts (CyPE and SuPE, respectively), and corresponding peptide amino acid sequences and spectral counts were retrieved from this previous proteomics survey. The selection of adhesion-related proteins for the subsequent analyses was based on their abundance (NSAF values) in *M. hyopneumoniae* 7448 SuPE.

2.2. Peptide validations, peptide abundance data, and protein topology and antigenicity predictions

To identify potential adhesin proteoforms in the selected adhesins, an initial validation of the CyPE- and SuPE-detected peptides corresponding to each adhesin was performed. For that, only adhesin corresponding peptides detected in at least two out of three biological replicates were considered as valid. The relative abundance of each valid peptide of a given protein was inferred from the correspondent average of that peptide's exclusive unique spectral counts in the biological replicates. Topology and antigenicity predictions were carried out using the whole deduced amino acid sequence of the selected proteins, retrieved from Uniprot database (https://www.uniprot.org/). Protein topologies were predicted using the Protter software (http://wlab.ethz.ch/protter/start/). Epitope predictions were performed using the "Predicted antigenic peptides" tool (http://imed.med.ucm.es/Tools/antigenic.pl).

2.3. Qualitative mapping of peptides and potential adhesin proteoform definition

For qualitative mapping of the CyPE and SuPE validated peptides into their correspondent proteins, the amino acid sequences of the *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare* ortholog adhesins of interest were aligned using the AliView software, with Muscle default parameters [24]. Validated peptides were then physically mapped into the cognate proteins. Topology and epitope predictions were taken into account to define extracellular location and antigenicity potential for all mapped peptides or peptide segments. A potential adhesin proteoform was defined as a predicted extracellular protein stretch comprehending two or more overlapping or juxtaposed mapped peptides, and with its amino and/or carboxy-terminal ends generated by non-tryptic cleavage events. Endoproteolytic cleavage sites were shown as 3 aa before and 3 aa after any experimentally (LC-MS/MS) mapped non-tryptic cleavage site. Endoproteolytic cleavage hotspots were defined as two or more experimentally (LC-MS/MS) determined cleavage sites < 10 aa apart.

2.4. Analyses of endoproteolytic processing regions

The non-tryptic ends of the identified proteoforms and their flanking 10 amino acid sequences (5 upstream and downstream amino acids), corresponding to endoproteolytic processing target-regions, were used to investigate the nature of mycoplasma endoproteolytic processing sites. For that, conservation of the target-regions was analyzed for each adhesin sets from *M. hyopneumoniae* strain or for *M. flocculare*, being the amino acid sequences of the target-regions aligned using the AliView software, with Muscle default parameters [24].

3. Results

3.1. M. hyopneumoniae and M. flocculare selected adhesion-related proteins and corresponding MS-based peptide identification

Adhesins detected by LC-MS/MS [9] in *M. hyopneumoniae* 7448 strains were ranked according to their abundances in the SuPE (Table S1). From the total of 35 identified adhesins, the top 5 most abundant ones were selected for further analyses, along with their orthologs in *M. hyopneumoniae* J and in *M. flocculare* (Table 1). For *M. hyopneumoniae* J, the selected ortholog set also corresponded to the top 5 most represented adhesins in SuPE, although the ranking was different for two of them. For *M. flocculare*, there was no ortholog for one of the selected adhesins (p97-copy 1), and the most represented one (DnaK) was the same ranked as such in *M. hyopneumoniae*. The other 3 orthologs were ranked in other ranking positions among the *M. flocculare* set of adhesins, in comparison to that of *M. hyopneumoniae*. Data regarding the selected ortholog adhesins, as their validated peptide sequences, peptide abundances, protein coverages and abundances, are described in Table S2.

3.2. Differential adhesin proteoforms found in M. hyopneumoniae and M. flocculare cell surface

Considering the previously described endoproteolytic processing of other *M. hyopneumoniae* adhesins [13,17,18,22], this issue was investigated for the selected set of top 5 most abundant *M. hyopneumoniae* 7448 adhesion-related proteins and in their *M. hyopneumoniae* J and *M. flocculare* orthologs. Tryptic and semi-tryptic peptide mapping was

Table 1
Top 5 most abundant adhesins and adhesion-related proteins in *M. hyopneumoniae* 7448 SuPE and corresponding orthologs in *M. hyopneumoniae* J and *M. flocculare*.

Protein name	*M. hyopneumoniae* 7448				*M. hyopneumoniae J*				*M. flocculare*			
	Ranking[a]	Accession number[b]	Coverage[c]		Ranking[a]	Accession number[b]	Coverage[c]		Ranking[a]	Accession number[b]	Coverage[c]	
			SuPE	CyPE			SuPE	CyPE			SuPE	CyPE
Chaperone protein DnaK (Heat shock 70 kDa protein)	1	**MHP7448_0067**	49.47%	79.27%	1	**MHJ_0063**	55.63%	83.83%	1	**MFC_01167**	47.47%	80.17%
46 kDa surface antigen (p46)	2	**MHP7448_0513**	54.23%	76.60%	2	**MHJ_0511**	59.77%	75.43%	8	**MFC_00037**	45.03%	68.83%
Protein P97-copy 1	3	**MHP7448_0198**	59.50%	74.07%	4	**MHJ_0194**	57.63%	68.57%	NE[d]	NE[d]	NE[d]	NE[d]
Putative p216 surface protein	4	**MHP7448_0496**	52.67%	77.87%	3	**MHJ_0493**	57.93%	72.67%	9	**MFC_00848**	50.37%	77.67%
ABC transporter xylose-binding lipoprotein	5	**MHP7448_0604**	36.43%	63.27%	5	**MHJ_0606**	38.63%	50.10%	2	**MFC_00388**	49.03%	65.10%

[a] According to LC-MS/MS abundance data in SuPE (Paes et al., 2018).
[b] Accession numbers retrieved from NCBI database (https://www.ncbi.nlm.nih.gov/protein).
[c] According to LC-MS/MS coverage data in CyPE and SuPE (Paes et al., 2018).
[d] NE, non existent.

performed for each adhesin of the ortholog sets detected in SuPE. Non-tryptic endoproteolytic cleavages were considered as the results of the activity *M. hyopneumoniae* or *M. flocculare* endogenous proteases. These mappings revealed from none to 13 endoproteolytic events, depending on the considered protein. Moreover, at least part of the identified endoproteolytic events were differential between strains and/or species, generating alternative proteoforms.

For the DnaK chaperone, the most represented adhesion-related protein in both *M. hyopneumoniae* and *M. flocculare*, proteoforms were identified only for the *M. hyopneumoniae* J ortholog (**MHJ_0063**), in which endoproteolytic processing resulted in the excision of a N-terminal sequence with 14 amino acids (Fig. 1 and Fig. S1). Comparisons between aligned ortholog DnaK amino acid sequences (Fig. 1C) demonstrated that the cleavage site found in *M. hyopneumoniae* J DnaK (G-T-T↓N-S-V) is conserved in both *M. hyopneumoniae* 7448 DnaK (**MHP7448_0067**) and *M. flocculare* DnaK (**MFC_01167**). However, no peptides corresponding to the N-terminal ends of the *M. hyopneumoniae* 7448 and *M. flocculare* orthologs were detected by LC-MS/MS.

For the p46 antigen orthologs from *M. hyopneumoniae* 7448 and J, and *M. flocculare* (**MHP7448_0513**, **MHJ_0511**, and **MFC_00037**, respectively), comparisons among the peptide mappings demonstrated that these proteins were differentially processed, potentially resulting in differential proteoforms among *M. hyopneumoniae* 7448 and J, and *M. flocculare*, (Fig. 2 and Fig. S2). Amino acid sequence alignments of non-tryptic cleavage site of *M. hyopneumoniae* 7448 and J p46 orthologs demonstrated that only the cleavage site Q-R-I↓Q-S-F was conserved (Fig. 2B). The other three cleavage events evidenced in *M. hyopneumoniae* J, apparently do not occur in strain 7448 or in *M. flocculare* (Fig. 2B). Despite the conservation of the predicted cleavage sites in *M. hyopneumoniae* 7448 and *M. flocculare*, the peptide mapping did not provide any evidence of cleavage. In *M. flocculare* p46, two different nested cleavage events within a 2-aa distance were observed (Fig. S2B), suggesting that this could be an endoproteolytic processing hotspot, not observed in *M. hyopneumoniae*.

For the p97 adhesin orthologs from *M. hyopneumoniae* 7448 and J (**MHP7448_0198** and **MHJ_0194**, respectively), the comparative peptide mapping indicated four and five non-tryptic cleavage sites, respectively, with sharing of two of these sites (Fig. S3). Only the cleavage sites Y-F-L↓N-F-E and I-K-S↓D-G-F were conserved in both p97 orthologs (Fig. S3C). The other 2 or 3 cleavage sites, differential between **MHP7448_0198** and **MHJ_0194**, could determine several other additional p97 adhesin proteoforms in each strain.

For the p216 adhesin orthologs from *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare* (**MHP7448_0496**, **MHJ_0493**, **MFC_00848**, respectively), the comparative peptide mapping revealed an even more complex scenario involving multiple and differential cleavage sites (Fig. S4) than that of p46 and p97 adhesins (see Fig. 2, Fig. S2, and Fig. S3). *M. hyopneumoniae* 7448 p216 presented eight cleavage sites, five of them shared with *M. hyopneumoniae* J (S-V-A↓F-K-P; L-L-Y↓P-G-V; N-Q-A↓V-E-N; G-L-Q↓S-F-Y; and E-R-S↓I-G-V) (Fig. S4D). *M. hyopneumoniae* J still presented other additional 8 differential cleavage sites (Fig. S4B). For *M. flocculare* p216, 12 cleavage sites were found, with a single one shared only with *M. hyopneumoniae* J (F-G-L/V↓L-Y-P) (Fig. S4B, C and D). The previously described cleavage in the T-N-F↓Q-E motif of the p216 adhesin [17] was confirmed by our data only in *M. hyopneumoniae* 7448. *M. hyopneumoniae* J and *M. flocculare* p216 orthologs also presented this conserved cleavage site their amino acid sequences, but no peptides covering or overlapping these sequences were detected in our LC-MS/MS analyses. Interestingly, p216 orthologs also presented one or two sites with nested endoproteolytic cleavages (hotspots), not conserved among them (Fig. S4).

For the *M. hyopneumoniae* 7448 and J, and *M. flocculare* orthologs of the ABC transporter xylose-binding lipoprotein (ATXBL) adhesion-related protein (**MHP7448_0604**, **MHJ_0606** and **MFC_00388**), the comparative peptide mapping revealed one endoproteolytic cleavage site in *M. hyopneumoniae* 7448 and J, and two in *M. flocculare* (Fig. 3 and Fig. S5). Endoproteolytic cleavage site K-N-V/I↓W-V-L was found in *M. hyopneumoniae* 7448 and *M. flocculare* (Fig. 3B), potentially generating N-terminal proteoforms with ~71% of similarity of amino acid sequence. This site is also found in the *M. hyopneumoniae* J ATXBL, but no peptides covering or overlapping it were mapped. Moreover, *M. hyopneumoniae* J and *M. flocculare* share an endoproteolytic hotspot, overlapping L-A-V↓A-G-P and G-P-L↓T-E-I cleavage sites (Fig. 3B).

Overall, the comparative peptide mapping of the selected ortholog adhesins from *M. hyopneumoniae* pathogenic and non-pathogenic strains, and *M. flocculare* provided evidence of the occurrence of extensive and differential endoproteolytic processing in these proteins. With that different adhesin proteoforms may be produced, with implications for the differential pathogenicity status of *M. hyopneumoniae* and *M. flocculare*.

3.3. Differential proteolytic processing between CyPE- and SuPE-detected adhesins

Comparisons between peptide mappings of adhesins detected in both CyPE and SuPE samples also provided evidences of differential proteolytic processing between subcellular compartments. For all adhesins of all assessed species and strains, most of the cleavage events observed in SuPE peptide mappings were not observed in CyPE ones, and vice-versa, as follows (Table S2, Fig. 1 and Figs. S6–S10).

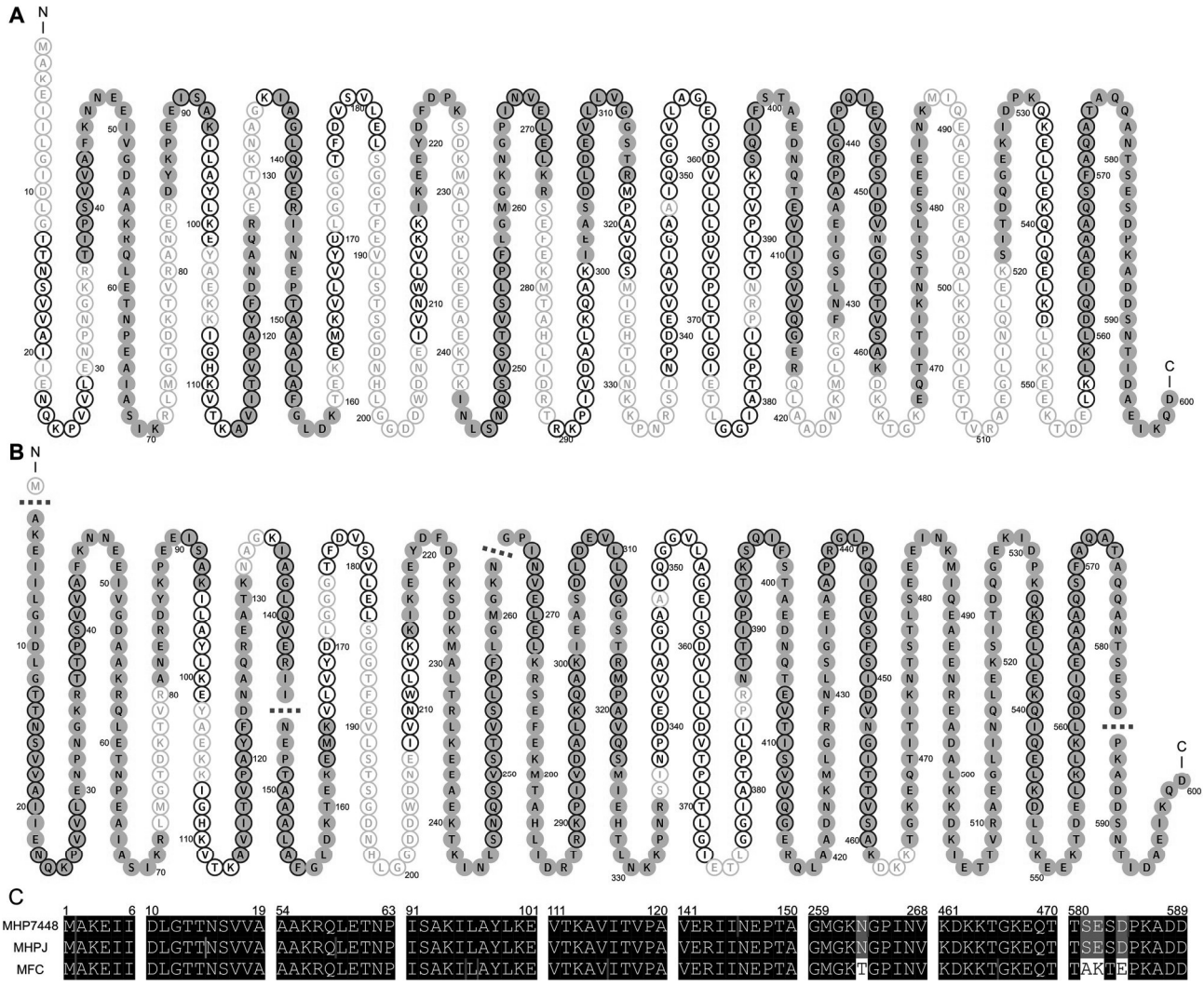

Fig. 1. *M. hyopneumoniae* 7448 DnaK chaperone (**MHP7448_0067**) detected peptides, identified non-tryptic cleavage events, and predicted epitopes. Panels A and B present the DnaK amino acid sequence with the peptides and non-tryptic cleavage events mapped into SuPE **(A)** and CyPE **(B)**. In these panels, colored circles (coral) indicate peptides detected by LC-MS/MS, according to Table S2; white circles indicate protein segments not detected by LC-MS/MS; non-tryptic cleavage events are indicated by dashed blue lines; red-framed circles indicate the predicted epitopes; N- and C-terminal ends are indicated, and amino acid residue positions are numbered. **(C)** Amino acid sequence alignments of endoproteolytic processing target regions of DnaK orthologs from *M. hyopneumoniae* 7448 (MHP7448) and J (**MHJ_0063**, MHPJ), and *M. flocculare* (**MFC_01167**, MFC). Initial and final amino acid residue positions defining the targeted regions are numbered, and endoproteolytic cleavage events are indicated by colored vertical bars, with orange ones corresponding to cleavage events detected in SuPE; and blue ones corresponding to cleavage events detected in CyPE. Mappings of detected peptides, identified non-tryptic cleavage events, and predicted epitopes of MHJ and MFC DnaK orthologs are shown in Fig. S1, for SuPE, and S6, for CyPE.

For DnaK chaperones, **MHP7448_0067** (Fig. 1B) and **MFC_01167** (Fig. S6B) orthologs presented four differential proteolytic cleavages in CyPE, but no evidences of non-trypic proteolytic events were found for them in SuPE. For the *M. hyopneumoniae* J ortholog, **MHJ_0063**, (Fig. S6A) on the other hand, a single endoproteolytic event was observed in CyPE (K-R-Q↓L-E-T), resulting in the excision of a 60-aa N-terminal protein fragment, instead of the 14 aa-excision observed in SuPE (see subsection 3.2 above). Alignments of ortholog DnaK amino acid sequences (Fig. 1C) demonstrated that all mapped cleavage sites are conserved among the orthologs, although the endoproteolytic cleavage events were strain/species-specific. Despite of these differences in proteolytic processing, all ortholog DnaK chaperones presented evidence of N-terminal methionine excision in CyPE.

The p46 antigen orthologs **MHP7448_0513**, **MHJ_0511**, and **MFC_00037** presented a conserved cleavage event (K-A-I/V↓G-S-K) in CyPE, which was not identified in SuPE (Figs. S7 and 2B, respectively). One additional cleavage event (mapped in a N-K-D↓P-A-G site) was found only for the *M. hyopneumoniae* J **MHJ_0511** ortholog, in both CyPE and SuPE. This finding of the same cleavage products in both CyPE and SuPE is suggestive that the responsible cleavage event occurs primarily in cytoplasm, being the resulting ends also presented in the surface. Moreover, considering that the K-A-I/V↓G-S-K cleavage event was the only one observed in the CyPE for *M. hyopneumoniae* 7448 and *M. flocculare* p46 orthologs, it can be assumed that these mycoplasmas share p46 cytoplasmic proteoforms, although their surface p46 proteoforms are differential (see subsection 3.2 above).

Regarding p97 orthologs, *M. hyopneumoniae* 7448 **MHP7448_0198** had four endoproteolytic cleavage sites mapped in each cellular compartment, but all of them were differential between CyPE and SuPE (Fig. S8 and Fig. S3, respectively). This finding demonstrated the presence of different *M. hyopneumoniae* 7448 p97 proteoforms in cytoplasm and surface. The *M. hyopneumoniae* J **MHJ_0194** ortholog, in turn, presented a single cleavage site (K-T-E↓Y-L-P) that was mapped for both CyPE and SuPE, although, as described above, four other additional cleavage sites were mapped in SuPE, two of them shared with the **MHP7448_0198** ortholog. Interestingly, this K-T-E↓Y-L-P cleavage

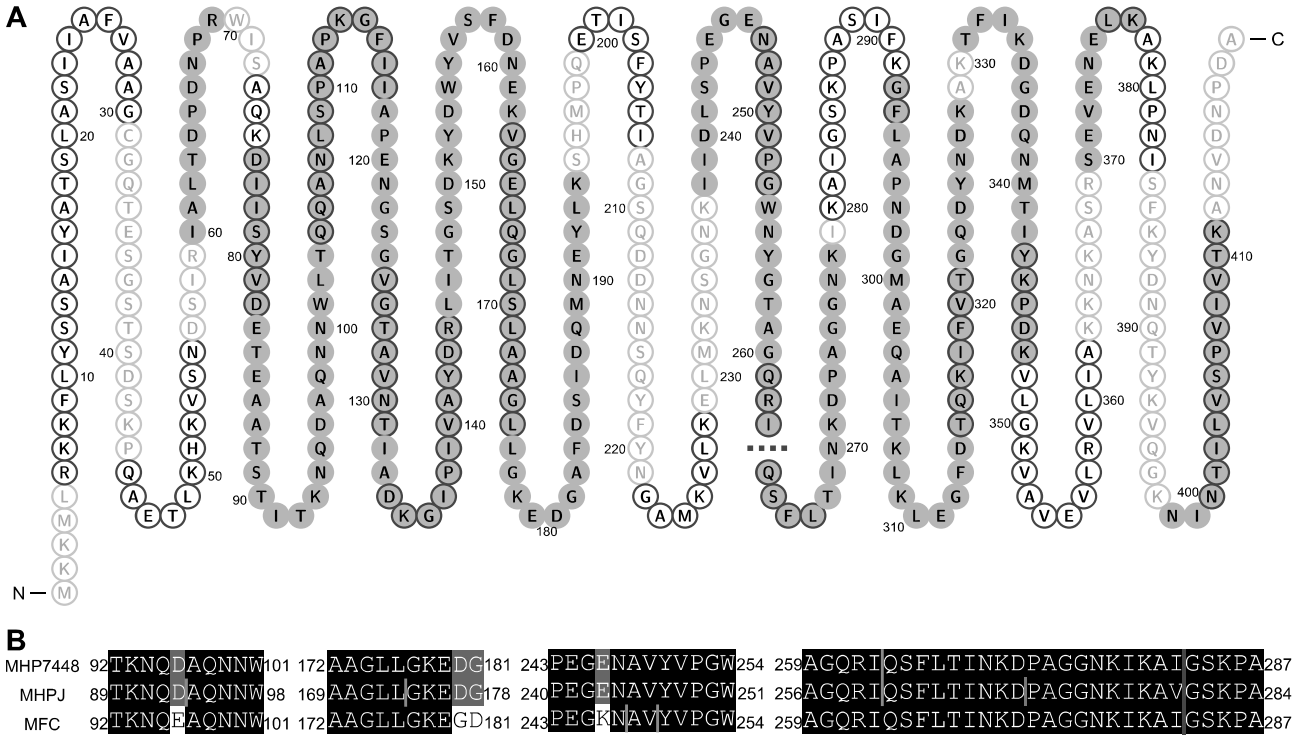

Fig. 2. *M. hyopneumoniae* 7448 p46 antigen (**MHP7448_0513**) detected peptides, identified non-tryptic cleavage events, and predicted epitopes. **(A)** P46 antigen amino acid sequence with the peptides and non-tryptic cleavage events mapped into SuPE. In this panel, colored circles (coral) indicate peptides detected by LC-MS/MS, according to Table S2; white circles indicate protein segments not detected by LC-MS/MS; non-tryptic cleavage events are indicated by dashed blue lines; red-framed circles indicate the predicted epitopes; N- and C-terminal ends are indicated, and amino acid residue positions are numbered. **(B)** Amino acid sequence alignments of endoproteolytic processing target regions of p46 antigen orthologs from *M. hyopneumoniae* 7448 (MHP7448) and J (**MHJ_0511**, MHPJ), and *M. flocculare* (**MFC_00037**, MFC). Initial and final amino acid residue positions defining the targeted regions are numbered, and endoproteolytic cleavage events are indicated by colored vertical bars, with orange ones corresponding to cleavage events detected in SuPE; blue ones corresponding to cleavage events detected in CyPE; and green ones corresponding to those detected in both SuPE and CyPE. Mappings of detected peptides, identified non-tryptic cleavage events, and predicted epitopes of MHJ and MFC p46 orthologs are shown in Fig. S2, for SuPE, and Fig. S7, for CyPE.

event was also mapped for the **MHP7448_0198** in CyPE, but not in SuPE.

For p216 orthologs, comparisons between CyPE and SuPE (Fig. S9 and Fig. S4, respectively) peptide mappings revealed that this adhesin is extensively processed in *M. hyopneumoniae* and *M. flocculare* cell surface, but not in the cytoplasm. From 8 (in *M. hyopneumoniae* 7448) to 13 and 12 (for *M. hyopneumoniae* J and *M. flocculare*, respectively) cleavage events were mapped in SuPE (see subsection 3.2 above), while from none (in *M. hyopneumoniae* J) to two (in both *M. hyopneumoniae* 7448 and *M. flocculare*) were mapped in CyPE. Interestingly, the previously described cleavage in the T-N-F↓Q-E motif [17] was mapped for *M. hyopneumoniae* 7448 in both CyPE and SuPE, suggesting that corresponding cleavage event occurs primarily in cytoplasm, as in the case of N-K-D↓P-A-G cleavage of the *M. hyopneumoniae* J p46 ortholog (described above).

ATXBL orthologs were also more extensively processed in *M. hyopneumoniae* and *M. flocculare* cell surface than in the cytoplasm. No cleavage sites were mapped in CyPE for **MHP7448_0604** and **MHJ_0606**, while one was mapped for **MFC_00388** (Fig. S10). On the other hand, from one (in *M. hyopneumoniae* 7448) to 3 (for *M. hyopneumoniae* J and *M. flocculare*) cleavage events were mapped in SuPE (see 3.2 subsection above). It is also noticeable, that the single cytoplasmic cleavage event (K-N-I/V↓W-V-L) mapped for the *M. flocculare* ATXBL ortholog was also mapped in SuPE for the *M. hyopneumoniae* 7448 and *M. flocculare* orthologs.

3.4. Analysis of endoproteolytic processing target-regions of surface adhesins

To search for possible consensual sequences among the target regions of mapped endoproteolytic events within each strain or species, the respective adhesin sets were separately compared. For these comparative analyses, target regions were defined as the five amino acid upstream and downstream of the non-tryptic end. All the target regions from each of the three assessed adhesin sets (from *M. hyopneumoniae* 7448, *M. hyopneumoniae* J and *M. flocculare*) were then aligned and compared. Most of the compared endoproteolytic processing regions presented low similarity between them. However, *M. hyopneumoniae* 7448 p97 and p216 adhesins share two endoproteolytic processing regions (Q-x-N-x-L/A↓V-x-x-F/A-x and x-S/N-x-x-L↓N/S-x-D-F) with 50–60% of similarity, respectively. Additionally, *M. hyopneumoniae* J p46 and ATXBL share an endoproteolytic processing region with 70% of similarity (A/V-A-G-L/P-L↓G/T-x-x-x-G/S), while 60% of this region is also conserved between p97 and p46 (x-x-Y/G-F/L-L↓N/G-x-E-D-x). The high similarities observed among endoproteolytic processing sites of some adhesins from *M. hyopneumoniae* suggests that these proteins may be proteolytic processed by the same protease at least in these conserved cleavage sites. On the other hand, no consensus endoproteolytic processing regions were observed among the *M. flocculare* adhesins. Overall, the low similarity found among most of the cleavage sites observed for *M. hyopneumoniae* strains and *M. flocculare* indicated that the analyzed adhesins may be targeted by different proteases.

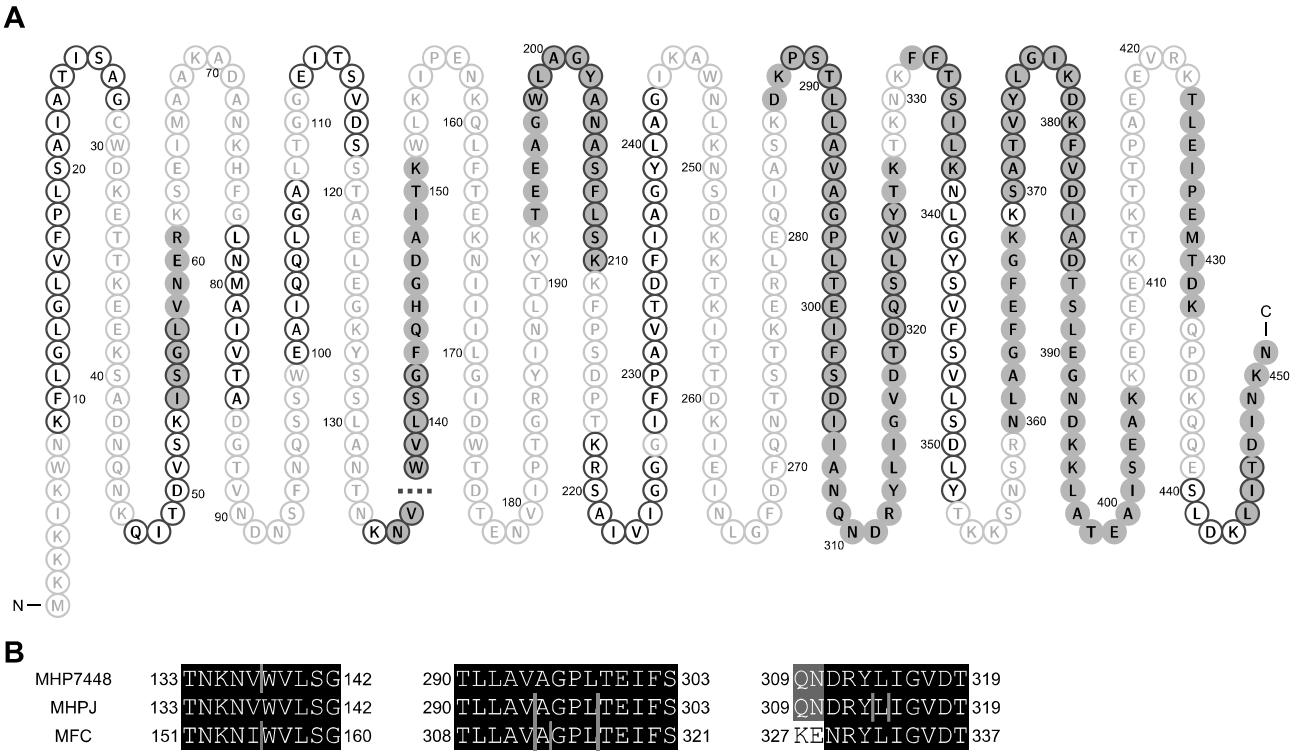

Fig. 3. *M. hyopneumoniae* 7448 ABC transporter xylose-binding lipoprotein (ATXBL) (**MHP7448_0604**) detected peptides, identified non-tryptic cleavage events, and predicted epitopes. **(A)** ATXBL amino acid sequence with the peptides and non-tryptic cleavage events mapped into SuPE. In this panel, colored circles (coral) indicate peptides detected by LC-MS/MS, according to Table S2; white circles indicate protein segments not detected by LC-MS/MS; non-tryptic cleavage events are indicated by dashed blue lines; red-framed circles indicate the predicted epitopes; N- and C-terminal ends are indicated, and amino acid residue positions are numbered. **(B)** Amino acid sequence alignments of endoproteolytic processing target regions of ATXBL orthologs from *M. hyopneumoniae* 7448 (MHP7448) and J (**MHJ_0606**, MHPJ), and *M. flocculare* (**MFC_00388**, MFC). Initial and final amino acid residue positions defining the targeted regions are numbered, and endoproteolytic cleavage events are indicated by colored vertical bars, with orange ones corresponding to cleavage events detected in SuPE; and green ones corresponding to those detected in both SuPE and CyPE. Mappings of detected peptides, identified non-tryptic cleavage events, and predicted epitopes of MHJ and MFC ATXBL orthologs are shown in Fig. S5, for SuPE, and Fig. S10, for CyPE.

3.5. Differential antigenicity of M. hyopneumoniae and M. flocculare adhesin proteoforms generated by endoproteolytic processing

After peptide mapping, identification of endoproteolytic cleavage events and the definition of differential adhesin proteoforms between *M. hyopneumoniae* strains and *M. flocculare* orthologs, the distribution of predicted epitopes was investigated for each proteoform. Epitope predictions were performed for the whole proteins, and then the fractions of the mapped peptides of each ortholog that overlapped with predicted epitopes were determined. As expected, most of the predicted epitopes were shared among analyzed orthologs, due to their high amino acid sequence similarity. Overall mapped epitope fractions (*i.e.*, the percentage of predicted epitopes covered by mapped peptides) were also similar, ranging from 38% to 53%, for *M. hyopneumoniae* 7448 adhesins, from 33% to 48%, for *M. hyopneumoniae* J adhesins, and from 35% to 52%, for *M. flocculare* adhesins. Despite of these overall similarities, the differential adhesin proteoforms expected from the mapped endoproteolytic processing events would bear different epitope distributions (see Figs. 1–3 and Figs. S1–S10). Comparisons of mapped epitope fractions among orthologs demonstrated that almost all *M. hyopneumoniae* 7448 adhesins bear larger fraction of mapped epitopes than their counterparts from the non-pathogenic mycoplasmas. For instance, *M. hyopneumoniae* 7448 DnaK and p97 bear 43.2% and 53.3% of mapped epitopes, respectively, while their orthologs bear ~36% and 47%, respectively. The only exception is the p216 adhesin from *M. flocculare*, which presented a larger fraction of mapped epitopes (52%) than those of their orthologs from *M. hyopneumoniae* 7448 (46%) and from *M. hyopneumoniae* J (44%).

4. Discussion

Adhesins or adhesin-related proteins are among the most abundant proteins in *M. hyopneumoniae* and *M. flocculare* cell surface [9]. Moreover, it has been shown that *M. hyopneumoniae* strains can shape their cell surface topography by post-translational processing of adhesins into surface-displayed adhesin proteoforms [10–12,14,15,17–19]. In this context, the recent comparative analyses of the cytoplasmic and surface proteomes of *M. hyopneumoniae* 7448 and J strains and *M. flocculare* [9] allowed to investigate the possible differential endoproteolytic processing among adhesins orthologs from different strain and species, previously demonstrated for the p97 adhesin [21]. With that in hand, the generation of adhesin proteoforms were inferred based on the occurrence of non-tryptic cleavage events in adhesins detected in cytoplasmic and surface fractions.

The five most abundant adhesins or adhesion-related proteins found in *M. hyopneumoniae* 7448 strain and their corresponding orthologs in *M. hyopneumoniae* J and *M. flocculare* were analyzed. Altogether, these adhesins correspond to more than 25% of the overall protein surface content (with at least 96–120 proteins) of these species. For these five adhesin orthogroups, there were no significative abundance differences between orthologs in interspecies comparisons, but the ranking position of each protein varied when comparing its abundance in cytoplasmic- and surface-enriched fractions within any given strain or species [9]. For instance, the abundance ranking was DnaK > p46 > p97 > p216 > ATXBL, for *M. hyopneumoniae* 7448, while it was DnaK > ATXBL > p46 > p216, for *M. flocculare* (see Table 1). Therefore, although each of these adhesins is not differentially represented in *M. hyopneumoniae* strains, and *M. flocculare* (considering its overall

abundance), it can be differentially represented in the cell surface compartment.

The DnaK chaperone can be regarded as a classical moonlighting protein, as it can acts as a canonical chaperone, or as an adhesin [18,25]. Moreover, it was also described as an antigenic protein [26]. It was also demonstrated that DnaK is the most abundant protein for both *M. hyopneumoniae* 7448 and J proteomes, and that its abundance is not altered even in heat stress conditions [27]. DnaK is consistently found in large amounts in both cytoplasmic and surface *M. hyopneumoniae* samples, but it was more endoproteolytically processed in cytoplasm than in surface. Such differential endoproteolytic processing would generate a larger repertoire of intracellular DnaK proteoforms. It is known that prokaryotic DnaK chaperones have two functional domains: a N-terminal ATPase domain of ~40 kDa and a C-terminal substrate-binding domain of ~25 kDa, which are connected by a short linker [25]. Interestingly, two of the three differential cleavage events observed for *M. hyopneumoniae* 7448 and *M. flocculare* cytoplasmic DnaK orthologs were within the ATPase domain. It can be hypothesized that these cleavage events may be negatively regulating canonical DnaK chaperone functions, by inactivating its ATPase domain. Alternatively, it is possible that the generated DnaK proteoforms, which may be differential between the two species, exert so far unknown functions independent of the protein's ATPase activity. These two hypotheses for the regulation of DnaK functions in these mycoplasmas are not mutually exclusive and deserve future investigation.

The p46 surface protein was initially characterized as an important *M. hyopneumoniae* antigen [28], but recently it has also been defined as an adhesin, based on its ability to bind some host extracellular matrix molecules, as fibronectin and heparin [18]. Our proteomic data demonstrated that the p46 antigen is also undergoing endoproteolytic processing, suggesting that different p46 proteoforms can be displayed in the cell surface. Most of the p46 mapped endoproteolytic cleavage events were differential among *M. hyopneumoniae* strains and *M. flocculare*, indicating that different portions of the antigen, with differential sets of predicted epitopes, can be presented to host immune system depending on the strain or species. Therefore, the presentation of differential p46 antigen proteoforms in the cell surface can be a mechanism of antigen variation, and contribute to immunomodulation, by eliciting differential immune responses against pathogenic or non-pathogenic *M. hyopneumoniae* strains, or against *M. flocculare*.

The ATXBL xylose ABC transporter is another recently identified as an adhesion-like protein [18]. It was shown that this protein was more endoproteolytically processed in the surface of *M. hyopneumoniae* J and *M. flocculare* than in *M. hyopneumoniae* 7448 surface, which could imply in a relatively larger loss of ATXBL function in orthologs from the non-pathogenic mycoplasmas. In this scenario, endoproteolytic cleavage events in *M. hyopneumoniae* J and *M. flocculare* ATXBL orthologs may differentially regulate the xylose uptake of these mycoplasmas in the swine host. On the other hand, the demonstrated ATXBL endoproteolytic processing may be responsible for the generation of diverse proteoforms with different adhesion properties or with alternative and so far unknown functions.

Considering canonical adhesins, the p97-copy 1 adhesin, which is exclusively present in *M. hyopneumoniae* strains [7], and the p216 adhesin, shared by *M. hyopneumoniae* and *M. flocculare*, have been extensively studied as *bona fide* adhesion-related proteins [14,15,17,19,29]. Moreover, they have also been studied regarding endoproteolytic processing and ectodomain shedding in *M. hyopneumoniae* 232 and J strains [14,15,17]. For p97, 1DE- and 2DE-LC-MS/MS data, showed that it is processed in multiple cleavage sites, generating protein fragments variating from 22 to 94 kDa [14,15]. Unfortunately, none of the previously described four cleavage sites p97 were covered by our proteomics analyses, as no semi-tryptic peptides covering or adjacent to them were detected, do not allowing more direct comparisons. This was possibly due to differences in the proteomic approaches, which include different cell fractioning methods and protein digestion conditions). On the other hand, our proteomic approach and subsequent data analyses allowed to identify novel endoproteolytic cleavage events for p97-copy 1 in both *M. hyopneumoniae* 7448 and J. This provided additional evidences of the generation of differential p97 proteoforms between *M. hyopneumoniae* pathogenic and non-pathogenic strains, as previously shown by Pinto et al. [21]. The identification of novel p97-copy 1 cleavage sites and consequent prediction of more putative proteoforms is in line with previous immunoblot analyses [14] that revealed multiple reactive protein bands corresponding to p97 paralogs.

For p216, a dominant cleavage event, consistently generating major proteoforms of 85 kDa and 120 kDa, was previously identified in both *M. hyopneumoniae* 232 and J strains [17]. This cleavage event was also mapped into *M. hyopneumoniae* 7448 p216 ortholog in both cytoplasmic and surface fractions, indicating that this cleavage site is targeted by an intracellular protease. Our proteomic data also demonstrated that p216 is cleaved in at least 8 and 13 different sites (in *M. hyopneumoniae* 7448 and J, respectively), which is in line with a previous demonstration that this adhesin, as p97 adhesins, can be further and extensively endoproteolytically processed, generating multiple proteoforms [17,19]. Moreover, our proteomic data showed that, except for the main cleavage event discussed above, the additional *M. hyopneumoniae* and *M. flocculare* p216 mapped cleavage sites are predominantly targeted in the cell surface, where most differential cleavage events were mapped, in comparison to those mapped in the cytoplasm. Interestingly, two of the cleavage target-regions of p216 and p97 adhesins presented > 60% similar amino acid sequences, suggesting that these adhesins are targeted by the same protease.

Regarding protease activities responsible for the observed adhesin post-translational processing, some candidate proteolytic enzymes have emerged from previous studies. *M. hyopneumoniae* strains and *M. flocculare* have from 14 to 18 genes coding for proteases annotated in their genomes, with experimental assessment of proteolytic activity for at least 7 of them (in *M. hyopneumoniae*) [22,30–33]. About a third of the overall repertoire of annotated proteases in each strain or species is represented by aminopeptidases, including the methionine aminopeptidase (MetAP), the M42 glutamyl aminopeptidase, and the XAA-PRO aminopeptidase. Interestingly, our proteomic data provided evidences of methionine aminopeptidase processing on DnaK orthologs in cytoplasmic fractions of both *M. hyopneumoniae* and *M. flocculare*. All the cytoplasmic N-terminal DnaK mapped peptides started in the second amino acid (Ala), without the N-terminal methionine residue. In line with that, in our *M. hyopneumoniae* and *M. flocculare* proteomic analyses, MetAP was exclusively identified in cytoplasmic fractions. Moreover, the M42 glutamyl aminopeptidase and the XAA-PRO aminopeptidase were found as more abundant in the cytoplasm than in the cell surface [9]. Additionally, our peptide mappings provided evidences of some nested N-terminal cleavage events in endoproteolytic processing hotspots, which suggests the occurrence of an initial endoproteolytic event followed by processive clipping by aminopeptidase activity(s). This was observed for three proteins and was differential (regarding the processing site) between *M. hyopneumoniae* and *M. flocculare* orthologs. Moreover, the occurrence of aminopeptidase cleavage events in the *M. hyopneumoniae* J p216 adhesin was also described [17].

Along the aminopeptidase activities, our proteomics data provided evidences of endoproteolytic cleavage events, indicating that all the analyzed adhesins were also targets of endoproteases, preferably (and differentially) in the cell surface. In line with that, comparisons between protease orthologs demonstrated that three annotated endoproteases (the ATP-dependent protease binding protein, the Lon protease and the oligoendopeptidase F) were more abundant in *M. hyopneumoniae* 7448 surface than in the surface of *M. hyopneumoniae* J and/or *M. flocculare* [9]. On the other hand, some of the analyzed adhesins (p46, p216 and ATXBL) were more endoproteolytically processed in *M. hyopneumoniae* J or *M. flocculare* surfaces, in comparison to

M. hyopneumoniae 7448. The final outcomes of the presence of proteases in the surface (or cytoplasm) is difficult to predict, as they depend not only on the abundance of any given protease in a cellular fraction, but also on its activity (and possible regulation), and its access to or affinity for the targeted substrate(s).

Additionally, the different cleavage sites mapped in each adhesin and the low similarity among them indicated that several proteases can target a same protein in both cytoplasm and surface fraction, but with different efficiencies. Moreover, the association of any of the mapped cleavage sites/events to a specific protease activity remains elusive, as well as the definition of specific proteoform lengths. In this context, future studies, involving *in vitro* cleavage assays and/or top-down proteomics, will be necessary in order to start assessing the activities of the different *M. hyopneumoniae* proteases and their specific substrates, and the specific proteoforms generated by endoproteolytic cleavage events.

The demonstrated differential endoproteolytic processing of adhesins among *M. hyopneumoniae* strains and *M. flocculare* is expected to significantly contribute to pathogenicity (or apathogenicity). This differential processing could be part of mechanism(s) for adhesion regulation or for antigen variation (associated to immune evasion or immunomodulation), for instance. It has also been suggested that *M. hyopneumoniae* adhesin post-translational proteolytic processing could generate a combinatorial library of adhesive proteoforms displayed cell surface [15,19,29]. The differential endoproteolytic cleavage events identified between *M. hyopneumoniae* strains and *M. flocculare* here demonstrated for five major adhesins may act as a regulatory mechanism for adhesion modulation, generating several adhesin proteoforms in different frequencies, composing different combinatorial library of adhesive proteoforms for strains or species. Such mechanism could be associated to the differences of adhesion capacities previously observed for different *M. hyopneumoniae* strains and *M. flocculare* [4]. Comparative analyses of the binding properties of adhesin proteoforms from *M. hyopneumoniae* and *M. flocculare* are needed to evaluate their potential contribution to bacterial adhesion to host cells.

In addition to the differential adhesion capacities between *M. hyopneumoniae* and *M. flocculare*, it was demonstrated that these mycoplasmas, despite the similarities in their repertoires of surface proteins [7] elicit differential immune responses [34,35]. In this context, the differential endoproteolytic cleavage events in surface-displayed adhesins here demonstrated (and also possible for other proteins) would contribute to differential antigenic proteoform presentation in the extracellular milieu. The resulting antigen variation may mediate immunomodulation or evasion from host immune responses.

The identification of endoproteolytic processing events and the subsequent definition of the generated adhesin proteoforms will be of interest for diagnostic and vaccinal epitopes. Two out the five analyzed *M. hyopneumoniae* adhesins (p46 and p97) have been studied regarding their vaccine and immunodiagnostic potential [26,36–38]. The results provided by our proteomics analyses are helpful to define well represented, surface-displayed epitopes from the analyzed adhesins. In this context, strain-specific epitopes from differential adhesin proteoforms are good candidates for new PEP immunodiagnostic tests, while species-specific epitopes could be used for recombinant vaccines development.

5. Conclusions

Overall, our analyses demonstrated that all the five analyzed adhesins undergo post-translational proteolytic cleavage events, and that several of them may be differential between cytoplasmic or in surface fractions. Moreover, most of the endoproteolytic cleavage events observed were poorly conserved among the analyzed adhesin orthologs, indicating that the occurrence of endoproteolytic processing is differential among *M. hyopneumoniae* strains and *M. flocculare*, further contributing for the formerly proposed combinatorial libraries of differential adhesin proteoforms displayed in cell surface. These adhesin proteoforms libraries may be determined by the different combination of protease activities targeting adhesin orthologs. Future structural and functional analyses of proteases and their target substrates are needed to elucidate the regulation mechanisms of differential proteolytic processing in *M. hyopneumoniae* and *M. flocculare* adhesins. The differential post-translational endoproteolytic processing of *M. hyopneumoniae* and *M. flocculare* adhesins are interesting for mycoplasma biology knowledge and can be taken as a base for new biotechnology applications.

CRediT authorship contribution statement

Lais Del Prá Netto Machado: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Jéssica Andrade Paes:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Priscila Souza dos Santos:** Formal analysis, Writing - original draft, Writing - review & editing. **Henrique Bunselmeyer Ferreira:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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5. DISCUSSÃO GERAL

Micoplasmas são bactérias de genoma reduzido, possuindo apenas algumas centenas de genes, que podem ser considerados os menores organismos capazes de auto-replicação. Esse pequeno repertório de genes poderia ser considerado um limitador de funções desempenhadas por esse organismo, devido ao pequeno número de opções de proteínas que poderiam ser codificadas. Porém, diferentes espécies de micoplasmas são capazes tanto de infectar uma extensa gama de animais, podendo causar infecções que persistem por longo prazo, por vezes, evadindo a resposta imune do hospedeiro por longos períodos; como podem ter uma relação de comensalismo com esses animais. Esses são os casos das duas linhagens de *M. hyopneumoniae* – a linhagem 7448, patogênica, e a linhagem J, não patogênica – e a linhagem comensal de *M. flocculare* tratadas nessa tese. *M. hyopneumoniae* são espécies geneticamente muito similares, possuindo genomas com >90% de compartilhamento de ortólogos com elevada conservação (SIQUEIRA *et al.*, 2013). Apesar disso, as diferenças evidenciadas em estudos genômicos e transcritômicos comparativos não foram capazes de explicar inteiramente a diferença de patogenicidade observada entre as linhagens/espécies aqui estudadas (ver, por exemplo, FERRARINI *et al.*, 2016; FERREIRA & DE CASTRO, 2007; SIQUEIRA *et al.*, 2013, 2014; STEMKE *et al.*, 1992; VASCONCELOS *et al.*, 2005)). Tendo em conta que estudos em nível de DNA e RNA não foram suficientes para explicar completamente as diferenças fenótipicas observada entre estas micoplasmas, especialmente aquelas associadas à patogênese ou virulência.

Nesta tese, foram utilizadas para abordagens proteômicas investigar possíveis diferenças quali-quatitativas nos repertórios de proteínas expressas por *M. hyopneumoniae* 7448 e J e *M. flocculare*, buscando associar tais diferenças à patogenicidade e à virulência. No Capítulo 1, foram caracterizados e comparados os repertórios de proteínas citoplasmáticas e de superfície nas linhagens e espécies aqui estudas. Procuramos diferenças entre as linhagens e espécies analisadas que pudessem ser relacionadas à patogenicidade. A partir das comparações feitas, foram evidenciadas diferenças quali-quantitativas nas amostras de *M. hyopneumoniae* 7448 em relação às amostras correspondentes de *M. hyopneumoniae* J e *M. flocculare*. Pela presença ou abundância diferencial em *M. hyopneumoniae* 7448, em especial na fração enriquecida com proteínas de superfície, as proteínas identificadas como diferenciais foram associadas à patogênese e à virulência desta

linhagem. Isso agrega conhecimento a dados prévios de estudos proteômicos do nosso grupo de pesquisa, que já evidenciaram outras diferenças entre estas espécies e linhagens em nível de repertórios proteicos (LEAL *et al.*, 2016; PAES *et al.*, 2017b, 2019; PINTO *et al.*, 2009).

Esta primeira parte da tese foi focada em proteínas de superfície, considerando a especial importância delas em processos de interação da bactéria com o hospedeiro como possíveis determinantes de patogenicidade e virulência. O sucesso do método de enriquecimento das amostras com proteínas de superfície, com a separação das frações solúvel e insolúvel, foi verificado com base na presença predominante, na fração enriquecida com proteínas de superfície, de proteínas preditas *in silico* como tal. O mesmo foi realizado para a confirmação do enriquecimento das frações solúveis, com proteínas preditas como citoplasmáticas.

No Capítulo 2, foi investigado o possível processamento proteolítico diferencial entre algumas das proteínas de superfície mais abundantes na superfície de *M. hyopneumoniae* 7448 em comparação a suas ortólogas de *M. hyopneumoniae* J e *M. flocculare*. Alguns estudos prévios mostraram que pelo menos algumas proteínas de *M. hyopneumoniae*, inclusive adesinas e outras proteínas possivelmente envolvidas com patogenicidade/virulência, são alvos de processamento pós-transcricional proteolítico (ASSUNÇÃO *et al.*, 2005; BERRY *et al.*, 2017; BOGEMA *et al.*, 2011, 2012; BURNETT *et al.*, 2006; DEUTSCHER *et al.*, 2012; DJORDJEVIC *et al.*, 2004; PINTO *et al.*, 2007; RAYMOND *et al.*, 2013, 2015; SEYMOUR *et al.*, 2010, 2012; TACCHI *et al.*, 2014, 2016; WIDJAJA *et al.*, 2017; WILTON *et al.*, 2009). Tal processamento aumenta as variantes (isoformas ou proteoformas) de proteínas apresentadas pela bactéria na sua superfície, conferindo maior variabilidade e aumentado a complexidade das interações da bactéria com o hospedeiro no contexto da PES. Este tipo de processamento pode gerar proteoformas com diferentes propriedades, que podem alterar processos como os de adesão e de imunomodulação, por exemplo.

Por ser de elevada complexidade, a abordagem de peptidômica escolhida foi aplicada à investigação apenas das cinco proteínas relacionadas à adesão mais abundantes na superfície na superfície de *M. hyopneumoniae* 7448 (conforme MACHADO *et al.*, 2020) em comparação a suas ortólogas de *M. hyopneumoniae* J e *M. flocculare*. Foram elas as adesinas p97 e p216, para as quais já havia evidência prévia de processamento proteolítico (DEUTSCHER *et al.*, 2010; DJORDJEVIC *et al.*, 2004; PINTO *et al.*, 2009; RAYMOND

et al., 2015; TACCHI *et al.*, 2014; WILTON *et al.*, 2009) e as proteínas DnaK, p46, e *ABC transporter xylose-binding lipoprotein*, já previamente relacionadas à adesão (GHAZAEI, 2017; TACCHI *et al.*, 2016) mas sem estudos quanto ao processamento proteolítico.

As análises peptidômicas feitas demonstraram que não só as adesinas bona fide p97 e p216, mas também a DnaK, p46, and *ABC transporter xylose-binding lipoprotein* são processadas proteoliticamente tanto em *M. hyopneumoniae* 7448 e J como em *M. flocculare*. Entretanto, a maioria dos eventos de clivagem detectados foram diferenciais entre as linhagens de *M. hyopneumoniae* e entre elas e *M. flocculare*, além de, em cada uma delas, serem também diferenciais entre as frações subcelulares citoplasmática e de superfície. Além disso, as análises *in silico* de antigenicidade realizadas apontaram para diferenças importantes nas proteoformas identificadas quanto aos seus repertórios de epítopos. Assim, o conjunto de resultados gerado evidenciou um cenário complexo, no qual eventos de processamento pós-traducional proteolítico diferenciais de proteínas de adesão contribuem para alterar o repertório de proteoformas e a arquitetura da superfície de linhagens de *M. hyopneumoniae* e de *M. flocculare*, provavelmente contribuindo com isso para virulência e patogenicidade.

A clivagem endoproteolítica, pode permitir, nas proteoformas geradas, a exposição de motivos ou domínios que, no enovelamento da proteína não processada, estariam encobertos ou em uma conformação não funcional. Uma vez expostos e em uma conformação adequada na superfície da bactéria, tais proteoformas, poderiam conferir propriedades diferenciais de adesão, imunomodulação ou outras, contribuindo significativamente para a patogênese, como determinantes de sinais clínicos da PES.

Por outro lado, as proteoformas diferenciais geradas por processamento pós-traducional proteolítico podem não somente proporcionar funções novas ou alternativas, mas também poderiam gerar proteoformas menos ou não funcionais. A falta de motivos ou domínios associados à patogenicidade ou virulência em proteoformas diferenciais de *M. hyopneumoniae* J ou *M. flocculare* poderiam contribuir para o caráter não patogênico destas linhagens e espécie. Futuros estudos imunológicos, bioquímicos e celulares com proteínas recombinantes correspondentes às diferentes proteoformas de *M. hyopneumoniae* 7448 e J e *M. flocculare* poderão evidenciar os papeis desempenhados por estas moléculas como fatores de virulência e determinantes ou não de patogenicidade.

6. CONCLUSÕES

Os resultados obtidos nesta tese forneceram um repertório abrangente do proteoma completo (citoplasmático e de superfície) celular de duas linhagens de *M. hyopneumoniae*, uma patogênica (7448) e outro não patogênica (J), e de *M. flocculare*. Dessa forma, fornecendo dezenas de possíveis determinantes de patogenicidade, muitos até então não classicamente descritos. Diversas classes de proteínas relacionadas com a virulência foram sobrerepresentadas em *M. hyopneumoniae* 7448, e incluem adesinas, proteases, proteínas relacionadas ao estresse oxidativo, transportadores de membranas, e proteínas com função *moonlightning,* juntamente com diversas proteínas de função desconhecida. Adicionalmente, o estudo das 5 adesinas mais representadas na linhagem patogênica, e 6 ortolólogos específicos nas não patogênicas, forneceram evidencias adicionais de eventos de clivagem endoproteolítica pós-traducional, muitos destes diferencias entre as frações de citoplasma e superfície. Assim, este trabalho evidenciou que eventos de clivagem diferencial entre as frações de superfície e citoplasmática e eventos pouco conservados entre os ortólogos entre micoplasmas patogênicos e não patogênicos. Esses resultados indicam que a ocorrência de processamento diferencial e abundâncias diferenciais de proteoformas de adesinas na superfície celular estão relacionados ao grau de patogenicidade de micoplasmas. Por fim, análises estruturais e funções das proteases e seus substratos alvo ainda são necessárias para elucidar/clarear o mecanismo de regulação do processamento proteolítico das adesinas de *M. hyopneumoniae* e *M. flocculare*.

7. PERSPECTIVAS

7.1 Clonar e expressar em *Escherichia coli* domínios extracelulares das proteoformas de adesinas e proteínas relacionadas com a adesão mais abundantes em *M. hyopneumoniae* 7448 e de seus ortólogos em *M. flocculare*;

7.2 Avaliar a antigenicidade e a imunogenicidade das proteoformas diferenciais de *M. hyopneumoniae* e *M. floculare*;

7.3 Avaliar propriedades de adesão das proteoformas diferenciais de *M. hyopneumoniae* e *M. floculare* a componentes da matriz extracelular e células ciliadas suínas;

7.4 Realizar co-cultivo de células do TRS e as proteoformas estudadas para avaliar possíveis alterações celulares, incluindo alterações morfológicas, efeitos citotóxicos e/ou indução de apoptose;

7.5 Realizar ensaios de inibição de adesão de *M. hyopneumoniae* a células suínas *in vitro* com anticorpos contra algumas das proteoformas estudadas;

7.6 Avaliar o potencial de algumas das proteoformas de proteínas relacionadas à adesão dominantes na superfície de *M. hyopneumoniae* como componentes vacinais para a prevenção da PES.

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XU, Jian *et al. Mycoplasma gallisepticum* MGA_0676 is a membrane-associated cytotoxic nuclease with a staphylococcal nuclease region essential for nuclear translocation and apoptosis induction in chicken cells. **Applied Microbiology and Biotechnology**, v. 99, n. 4, p. 1859–1871, 2015.

YAMAMOTO, Takeshi *et al.* Mpn491, a secreted nuclease of *Mycoplasma pneumoniae*, plays a critical role in evading killing by neutrophil extracellular traps. **Cellular Microbiology**, v. 19, n. e12666, p. 1–11, 2017. Disponível em: < https://onlinelibrary.wiley.com/doi/10.1111/cmi.12666 >.

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YU, Yanfei *et al.* Elongation factor thermo unstable (EF-Tu) moonlights as an adhesin on the surface of *Mycoplasma hyopneumoniae* by binding to fibronectin. **Frontiers in Microbiology**, v. 9, n. 974, p. 1–12, 2018a.

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ZHANG, Jing *et al.* Inter- and intra-strain variability of tandem repeats in Mycoplasma pneumoniae based on next-generation sequencing data. **Future Microbiology**, v. 12, n. 2, p. 119–129, 2017. Disponível em: < https://www.futuremedicine.com/doi/10.2217/fmb-2016-0111 >.

ZHANG, Hui *et al. Mycoplasma bovis* MBOV _ RS02825 Encodes a Secretory Nuclease Associated with Cytotoxicity. **International Journal of Molecular Sciences**, v. 17, n. 628, p. 1–18, 2016.

ZHANG, Q.; YOUNG, T. F.; ROSS, R. F. Identification and characterization of a *Mycoplasma hyopneumoniae* adhesin. **Infection and Immunity**, v. 63, n. 3, p. 1013–1019, 1995. Disponível em: < http://pmc/articles/PMC173103/?report=abstract >.

ZHANG, Qijing; YOUNG, Theresa F.; ROSS, Richard F. Microtiter Plate Adherence Assay and Receptor Analogs for *Mycoplasma hyopneumoniae*. **Infection and Immunity**, v. 62, n. 5, p. 1616–1622, 1994.

ZHAO, Ping *et al.* Identification of novel immunogenic proteins in *Mycoplasma capricolum* subsp. *capripneumoniae* strain M1601. **Journal of Veterinary Medical Science**, v. 74, n. 9, p. 1109–1115, 2012.

ZIELINSKI, G. C.; ROSS, R. F. Adherence of *Mycoplasma hyopneumoniae* to porcine ciliated respiratory tract cells. **American journal of Veterinary Research**, v. 54, n. 8, p. 1262–1269, 1993.

ZIELINSKI, G C; ROSS, R F. Effect of growth in cell cultures and strain on virulence of *Mycoplasma hyopneumoniae* for swine. **American journal of Veterinary Research**, v. 51, n. 3, p. 344–348, 1990.

ZIMMERMAN, Jeffrey J. *et al.* **Diseases of Swine**. 11th ed. Hoboken, NJ: Wiley-Blackwell, 2019. *E-book*. Disponível em: < https://onlinelibrary.wiley.com/doi/book/10.1002/9781119350927 >.

Curriculum Vitae resumido

DEL PRA NETTO MACHADO, L.; MACHADO, LAÍS DEL PRÁ NETTO; MACHADO, LD; MACHADO, LAÍS D. P. N.; MACHADO, LDPN; MACHADO, L DEL P; MACHADO, LAIS DEL PRÁ NETTO

1. DADOS PESSOAIS

Nome: Lais Del Prá Netto Machado

Local e Data de Nascimento: Blumenau, Santa Catarina, Brasil, 29/03/1989

Endereço Profissional:

Universidade Federal do Rio Grande do Sul, Centro de Biotecnologia
Avenida Bento Gonçalves, 9500 Prédio 43421 salas 204/206/210
91501-970 Porto Alegre, RS, Brasil

Telefone Profissional: (051) 33087769
E-mail: laisdelpra@gmail.com
laismachado.contato@gmail.com

2. FORMAÇÃO ACADÊMICA

2017 – 2022

Doutorado em andamento em Biologia Celular e Molecular
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brasil
Orientador: Henrique Bunselmeyer Ferreira
Co-orientadora: Jéssica Andrade Paes Vieira
Bolsista do: Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasil

2013 – 2015

Mestrado em Farmácia
Universidade Federal de Santa Catarina, UFSC, Florianópolis, SC, Brasil
Orientadora: Thaís Cristine Marques Sincera
Co-orientador: Caio Maurício Mendes de Cordova
Bolsista da: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil

2007 – 2011

Graduação em Farmácia (Habilitação em Bioquímica | Análises Clínicas)
Universidade Regional de Blumenau, FURB, Blumenau, SC, Brasil

3. ESTÁGIOS

2012 - 2012

Estágio Voluntário
Enquadramento Funcional: Pesquisadora
Carga horária: 30h/semana | Dedicação exclusiva
Duração: 6 meses (Abril/2012 – Setembro/2012)

Laboratório de infecções humanas por clamídia e micoplasma ("*Infections humaines à mycoplasmes et à chlamydiae*"), Hospital Pellegrin, Universidade de Bordeaux (*Université de Bordeaux*), Aquitaine, França.

Supervisora: Cécile Bébéar

Atividades: Pesquisa relacionada a caracterização da bactéria *Mycoplasma pneumoniae* tipo I e tipo II por espectrometria de massa com fonte de ionização e dessorção a laser assistida por

matriz (do inglês, *Matrix Assisted Laser Desorption/Ionization*, MALDI) e analisador de massas do tempo-de-voo. (do inglês, *Time-of-Flight*, TOF), conhecido como MALDI-TOF. Realização da parte experimental, cultura da bactéria, análise de amostras biológicas, padronização de protocolos, utilização de softwares específicos para o MALDI-TOF.

2012 - 2012

Estágio

Enquadramento Funcional: Estágio de Aperfeiçoamento

Carga horária: 16h

Iniciação a análise de sequências em bioinformática | 2012 - 1144/1 – UBX2 - *Initiation à l'analyse de séquences en bioinformat*

Universidade de Bordeaux (*Université de Bordeaux*), Aquitaine, França.

Supervisora: Céline Iacono Di Cacito

Atividades: Acesso a banco de dados de sequências de ácidos nucleicos e proteínas. Utilização de ferramentas (Softwares) e utilizadas dentro da bioinformática, como o BLAST (do inglês, *Basic Local Alignment Search Tool*) para alinhamento de sequências e desenho de iniciadores (*Primers*).

2011 - 2011

Estágio Curricular Supervisionado

Enquadramento Funcional: Estagiária

Área do Estágio	Carga Horária
Microbiologia e Imunologia clínica	144h
Hematologia e Citologia Clínica	144h
Bioquímica Clínica e Uroanálise	144h
Parasitologia e Micologia Clínica	144h

Carga horária: 576h

Laboratório de Análises Clínicas (LAC), FURB, Blumenau, SC, Brasil

Atividades: Realizar e interpretar exames laboratoriais clínicos, prestar serviço técnico-científico sobre os principais exames realizados, avaliar o uso e possíveis interferências de medicamentos e alimentos nos exames laboratoriais, realizar procedimentos relacionados a coleta de material, manipulação de amostras biológicas (manipulação, triagem, preparo) para fins de exames laboratoriais. Conhecimento aprofundado dos equipamentos, preparação de reagentes, calibradores, avaliação do controle de qualidade.

2010 - 2010

Estágio Curricular

Enquadramento Funcional: Estagiária

Carga horária: 180h

Farmácia Sesi – Unidade Escola Agrícula, Serviço Social da Indústria (SESI)

FURB, Blumenau, SC, Brasil

Atividades: Acompanhamento da rotina e atividades desenvolvidas pelo farmacêutico, como: aquisição, armazenamento, distribuição e dispensação de medicamentos e/ou produtos farmacêuticos. Adquirir conhecimento relacionados a assistência farmacêutica, assuntos regulatórios e administrativos, informações sobre medicações, educação em saúde.

2007 - 2007

Estágio Voluntário

Enquadramento Funcional: Estagiária

Período: 02/04/2007 – 02/06/2007

Laboratório de Microscopia – Histotécnica, FURB, Blumenau, SC, Brasil

Orientadora: Cláudia Almeida Coelho de Albuquerque

Atividades: Análise dos Parâmetros Hematológicos e Bioquimicos do Sangue de *Bothrops jararaca* e *Bothropus jararacussu* mantidas no Serpentário da Universidade Regional de Blumenau

4. CURSOS

Ano	Curso	Evento/Instituição	C.H.*
2020	PH125.1x: Data Science: R Basics	HarvardX, Harvard University, Estados Unidos	16h
2017	Introdução a Biologia de Sistema e Análise de Rede	2ª Ed. Da Escola Gaúcha de Bioinformática – EGB. UFRGS, Brasil.	15h
2007	Hematologia Clínica	2º Simpósio Catarinense de Análises Clínica. Sociedade Brasileira de Análises Clínicas, SBAC, Brasil.	8h
2007	Doença de Alzheimer: Patogenia e Farmacologia	II Semana da Modernidade: Neurociências. FURB, Brasil	17h

*Carga Horária

5. DISTINÇÕES

2019 – Palestrante. Curso de Férias | I Curso de Atualização de Professores - **Lais Del Prá Netto Machado** ministrou a palestra intitulada "Vacinas". Curso de extensão organizado pelo Programa de Pós-graduação em Biologia Celular e Molecular (PPGBCM), UFRGS, Brasil (Carga horária: 1h 30 min).

2016 – Participação de Banca Avaliadora em Trabalhos de Conclusão. MANFREDI, M. A. B.; Serdes, Adriana; **DEL PRA NETTO MACHADO, L**. Participação da banca de Shaiana Paula Pandini. Trabalho intitulado: Infecção do Trato Urinário associada ao Cateterismo Vesical. 2016. Trabalho de Conclusão de Curso (Graduação em Biomedicina) - Fundação Universidade Regional de Blumenau.

2014 – Organização de evento | Congresso. **Lais Del Prá Netto Machado** trabalhou como organizador voluntário do IOM | 20th *Congress of the International Organization for Mycoplasmology* – Blumenau, SC, Brasil. (Carga horária: 40h).

6. EXPERIÊNCIA PROFISSIONAL OU DIDÁTICA ANTERIOR

2016 – 2016

Farmacêutica

Enquadramento Funcional: Responsável Técnica Farmacêutica

Vínculo: Celetista

Período: 26/08/2016 – 23/09/2016

Farmácia Farmais Posto Universitário, Porto Alegre, RS, Brasil.

Atividades: Atendimento de clientes, organização de estoque, atenção farmacêutica, controle de medicamentos controlados (psicotrópicos e antibióticos), SNGPC, gerência da farmácia, controle de estoque.

2016 – 2016

Professora

Enquadramento Funcional: Professor Substituto; Dedicação Exclusiva

Enquadramento Profissional:

Professora (Teórica e Prática) de Bacteriologia Clínica II | Curso Biomedicina

Professora (Prática) de Bacteriologia Clínica I | Curso Biomedicina

Professora (Teórica e Prática) de Bacteriologia Clínica II | Curso Biomedicina

Período: 18/02/2016 – 31/07/2016

Instituição: Universidade Regional de Blumenau, Blumenau, SC, Brasil.

Atividades: Preparar e lecionar aulas teóricas e práticas, preparar e aplicar provas e exercícios. Avaliar alunos.

2015 – 2015

Professora

Enquadramento Funcional: Professor Substituto; Dedicação Exclusiva

Enquadramento Profissional:

Professora de Bioquímica Clínica I | Curso Técnico de Análises Clínicas

Período: 04/08/2015 – 22/12/2015

Professora de Microbiologia Clínica II | Curso Técnico de Análises Clínicas

Período: 03/09/2015 – 22/12/2015

Professora de Farmacologia | Curso Técnico de Enfermagem

Período: 04/08/2015 – 22/12/2015

Professora de Microbiologia e Parasitologia | Curso Técnico de Enfermagem

Período: 03/09/2015 – 22/12/2015

Instituição: Centro de Educação Profissional Hermann Hering (CEDUPHH)

Estado de Santa Catarina Secretaria de Estado da Educação | GERED – Gerência Regional de Educação 15ª SDR | CEDUP – Centro de Educação Profissional. Blumenau, SC, Brasil.

Atividades: Preparar e lecionar aulas das disciplinas teóricas e práticas. Preparar e aplicar provas e exercícios. Avaliar alunos.

2010 – 2011

Farmacêutica

Enquadramento Funcional: Responsável Técnica Farmacêutica

Vínculo: Celetista

Período: 04/08/2010 – 13/12/2011

Farmácia FarmaCelso, Blumenau, SC, Brasil

Atividades: Atendimento de clientes, organização de estoque, atenção farmacêutica, controle de medicamentos controlados (psicotrópicos e antibióticos), SNGPC, gerência da farmácia, controle de estoque.

7. ARTIGOS COMPLETOS PUBLICADOS

7.1. MACHADO, L. D. P. N.; PAES, J. A.; SOUZA, P. S.; FERREIRA, H. B. Evidences of differential endoproteolytic processing on the surfaces of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*. *Microbial Pathogenesis*. MICROBIAL PATHOGENESIS, v. 140, p. 103958, 2020.

7.2. PAES, JÉSSICA ANDRADE; MACHADO, LAIS DEL PRÁ NETTO; DOS ANJOS LEAL, FERNANDA MUNHOZ; DE MORAES, SOFIA NÓBREGA; MOURA, HERCULES; BARR, JOHN R.; FERREIRA, HENRIQUE BUNSELMEYER. Comparative proteomics of two

Mycoplasma hyopneumoniae strains and *Mycoplasma flocculare* identified potential porcine enzootic pneumonia determinants. Virulence, v. 9, p. 1230-1246, 2018.

7.3. CORDOVA, CAIO M.M.; HOELTGEBAUM, DANIELA L.; MACHADO, LAÍS D.P.N.; SANTOS, LARISSA DOS. Molecular biology of mycoplasmas: from the minimum cell concept to the artificial cell. ANAIS DA ACADEMIA BRASILEIRA DE CIÊNCIAS (ONLINE), v. 88, p. 599-607, 2016.

7.4. MACHADO, LAÍS DEL PRÁ NETTO; MOLINARI, MARCELO A.; DOS SANTOS, LARISSA; DE CORDOVA, CAIO M.M. Performance of four commercial kits for laboratory diagnosis of urogenital mollicute infection. CANADIAN JOURNAL OF MICROBIOLOGY, v. 60, p. 1-5, 2014.

7.5. SAMPAIO, J.; Camila Fernandes Ceola; DEL PRA NETTO MACHADO, L.; Caio Maurício Mendes de Cordova. Avaliação da Progressão da Imunodepressão em Pacientes com HIV/AIDS em relação à presença de Micoplasmas do Trato Urogenital. TENDÊNCIAS EM HIV AIDS, v. 8, p. 8-12, 2013.

7.6. PEREYRE, S.; TARDY, F.; RENAUDIN, H.; CAUVIN, E.; DEL PRA NETTO MACHADO, L.; TRICOT, A.; BENOIT, F.; TREILLES, M.; BEBEAR, C. Identification and Subtyping of Clinically Relevant Human and Ruminant Mycoplasmas by Use of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. Journal of Clinical Microbiology (Print), v. 51, p. 3314-3323, 2013.

8. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

8.1. Paes, J. A.; Leal, F. M. A.; Machado, L. D. P. N.; de Moraes, S. N.; Moura, H.; Barr, J.; Ferreira, H. B. Comparative proteomics of two *Mycoplasma hyopneumoniae* strains and *Mycoplasma flocculare* for the identification of novel porcine enzootic pneumonia determinants. 2018. Apresentação de Trabalho/Congresso). IOM | 22th Congress of the International Organization for Mycoplasmology – Portsmouth, Estados Unidos da América.

8.2. Machado, L. D. P. N.; Paes, J. A.; Leal, F. M. A.; Moura, H.; Barr, J.; Ferreira, H. B. Comparative proteomics analyses of *M. hyopneumoniae* e *M. flocculare* surface proteins. 2018. Apresentação de Trabalho/Congresso). IOM | 22th Congress of the International Organization for Mycoplasmology – Portsmouth, Estados Unidos da América.

8.3. Machado, Lais Del Pra Netto; Hoeltgebaum, Daniela Laiza; Sincero, Thaís Cristine Marques; Cordova, Caio M. M.; Role of *Mycoplasma pneumoniae*. 2016. (Apresentação de Trabalho/Congresso). IOM | 21th Congress of the International Organization for Mycoplasmology – Brisbane, Austrália.

8.4. Machado, Lais Del Pra Netto; Cordova, Caio M. M.; Sincero, Thaís Cristine Marques. *In vitro* comparison of previously designed primers and new primers designed with better *in silico* properties for *Mycoplasma pneumoniae* detection. 2016. (Apresentação de Trabalho/Congresso). IOM | 21th Congress of the International Organization for Mycoplasmology – Brisbane, Austrália.

8.5. Pereyre, S.; Tardy, F.; Renaudin, H; Cauvin, E.; **Del Prá Netto Machado, L.**; Tricot, A.; Benoit, F.; Tricot, A.; Treilles, M.; Bébéar, C. Identification and subtyping of clinically relevant human and ruminant mycoplasmas using matrix-assisted laser desorption ionization-time of flight mass spectrometry. 2014. (Apresentação de Trabalho/Congresso).

IOM | 20th Congress of the International Organization for Mycoplasmology – Blumenau, SC, Brasil.

8.6. **Machado, Lais Del Pra Netto;** Sincero, Thaís Cristine Marques; Cordova, Caio M. M. *In silico* analysis of previously designed primers for *Mycoplasma pneumoniae* detection and basis for design of new primers of better performance. 2014. (Apresentação de Trabalho/Congresso).

IOM | 20th Congress of the International Organization for Mycoplasmology – Blumenau, SC, Brasil.

8.7. **Machado, Lais Del Pra Netto Machado;** Sincero, Thaís Cristine Marques; Cordova, Caio M. M. *In silico* analysis of previously designed primers for *Mycoplasma pneumoniae* detection and basis for design of new primers of better performance. 2014. (Apresentação de Trabalho/Congresso).

IOM | 20th Congress of the International Organization for Mycoplasmology – Blumenau, SC, Brasil.

8.8. Hoeltgebaum, A. L.; Cordova, C. M. M.; **Machado, L. D. P. N.** Diagnóstico da Pneumonia Atípica Primária: Prevalência de *Mycoplasma pneumoniae*. 2014. (Apresentação de Trabalho/Outra).

MIPE | 8ª Mostra Integrada de Ensino, Pesquisa e Extensão – FURB, Universidade Regional de Blumenau, Blumenau, SC, Brasil.

8.9. **Machado, L. DEL P. N.;** Molinari, M. A.; Cordova, C. M. M. Performance of Commercial Kits Available in Brazil for Laboratory Diagnosis of Urogenital Mollicute Infection. 2012 (Apresentação de Trabalho/Congresso).

XXI ALAM | XXI Congresso Latinoamericano de Microbiologia – Mendes Conventional Center, Santos, SP, Brasil.