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

Caracterização das vias de resposta de células suínas durante a infecção com micoplasmas do trato respiratório suíno

Tese de Doutorado

Fernanda Munhoz dos Anjos Leal

Porto Alegre, 18 de outubro de 2018

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exemplos de mulheres fortes.
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SUMÁRIO

LISTA DE ABREVIATURAS

AT – adenina e timina

ATP – adenosine triphosphate

BHI – *brain heart infusion*

CC – componente celular

CCU – *colour change unit*

CDRS – complexo de doenças respiratórias suínas

CDS – sequência(s) de DNA codificadora(s)

CFU – *colony formation unit*

DAMPs – *damage-associated molecule patterns*

DNA – ácido desoxirribonucleico

ELISA – ensaio imunoenzimático indireto

EP – *enzootic pneumonia*

ER – *endoplasmic reticulum*

FA – *formic acid*

FBS – *fetal bovine serum*

FC – *fold change*

FDR – *false discovery rate*

FM – função molecular

GO – *gene ontology*

HPLC – *high performance liquid chromatography*

KEGG – Kyoto Encyclopedia of Genes and Genomes

LAMPs – *lipid-associate membrane proteins*

LC-MS/MS – *liquid chromatography tandem mass spectrometry*

MAPK – *mitogen-activated protein kinase*

NSAF – *normalized spectral abundance factor*

OMM – *outer mitochondrial membrane*

PB – processos biológicos

PBS – solução salina tamponada com fosfato

PCR – reação em cadeia da polimerase

PES – pneumonia enzoótica suína

PPLO – *pleuropneumonia like organism enrichment*

PRRSV – *porcine reproductive and respiratory syndrome virus*

ROS – *reactive oxygen species*

SDS – dodecilsulfato de sódio

SDS-PAGE – eletroforese em gel de poliacrilamida na presença de SDS

SEM – *Spiroplasma, Entomoplasma, Mesoplasma, Mycoplasma e Ureaplasma*

SFB – soro fetal bovino

SFM – *serum free medium*

TFA – *trifluoroacetic acid*

UPR – *terminal unfolded protein response*

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RESUMO

Mycoplasma hyopneumoniae é o agente etiológico da pneumonia enzoótica suína (PES), uma doença com distribuição global e considerada uma das principais causas de perdas econômicas na suinocultura. Os conhecimentos sobre a fisiologia de *M. hyopneumoniae* e de suas interações com o suíno ainda são limitados, porém foi demonstrado que a resposta do hospedeiro suíno contra *M. hyopneumoniae* possui papel fundamental no desenvolvimento da PES. Desse modo, para elucidar as interações bactéria-hospedeiro e identificar determinantes da PES, os proteomas de uma linhagem celular suína (NPTr) infectada com linhagens patogênicas e não-patogênicas de *M. hyopneumoniae* (7448 e J, respectivamente) e com a espécie geneticamente relacionada, *M. flocculare,* foram analisados por LC-MS/MS. A comparação das respostas da células NPTr, frente a cada micoplasma e de cada micoplasma frente às células hospedeiras indicaram possíveis novos mecanismos responsáveis pela patogênese de *M. hyopneumoniae*. Os resultados apresentados neste trabalho mostram uma resposta diferencial e específica das células NPTr à infecção com a linhagem de micoplasma patogênica *M. hyopneumoniae* 7448 quando comparada com as linhagens de micoplasmas não-patogênicas *M. hyopneumoniae* J e *M. flocculare*. Proteínas envolvidas com mecanismos de patogênese até então desconhecidos, como por exemplo, damage-associated molecule patterns (DAMPs) e terminal unfolded protein response (UPR), foram identificadas na resposta específica à *M. hyopneumoniae* 7448. Além disso, foi observada a secreção diferencial de fatores de virulência pelas linhagens de micoplasmas, bem como a presença de diversos fatores de virulência nas vesículas extracelulares, ressaltando a importância das proteínas secretadas para a patogênese de *M. hyopneumoniae*. Por fim, este trabalho forneceu uma extensa lista de proteínas suínas e de *M. hyopneumoniae* que são alvos interessantes para estudos adicionais na elucidação dos determinantes da PES e que podem permitir, futuramente, a descoberta de novos métodos para a prevenção, diagnóstico e tratamento da doença.

ABSTRACT

Mycoplasma hyopneumoniae is the etiological agent of enzootic pneumonia (EP), a widespread disease considered a major cause of economic loss in the pig industry. Limited knowledge is available about *M. hyopneumoniae* physiology and its interactions with swine host, however, it has been demonstrated the importance of the swine host response against *M. hyopneumoniae* in EP development. Thus, in order to elucidate bacterium-host interactions, and identify EP determinants, the proteomes of swine cell line, NPTr, infected with pathogenic e non-pathogenic *M. hyopneumoniae* strains and the genetically closed species, *M. flocculare,* were evaluated by LC-MS/MS. Comparisons of NPTr cells responses against each mycoplasma e from each mycoplasma indicated possible new mechanisms involved in *M. hyopneumoniae* pathogenesis. The results presented here showed a differential and specific response of NPTr cells against the pathogenic *M. hyopneumoniae* 7448 strain in comparison with non-pathogenic *M. hyopneumoniae* J or *M. flocculare* strains. Proteins involved with pathogenesis mechanisms, as DAMPs and UPR, were identified specifically in response to *M. hyopneumoniae* 7448. Moreover, it was observed differential mycoplasmas virulence factors secretion, including in extracellular vesicles, highlighting the importance of secreted proteins during *M. hyopneumoniae* pathogenesis. Finally, this study provided an extensive list of swine and *M. hyopneumoniae* proteins that are interesting targets for further studies about EP determinants. In the future, these targets can contribute to discovery of new methods to EP prevention, diagnosis and treatment.

1. INTRODUÇÃO

1.1. Características gerais de *Mycoplasma hyopneumoniae* e *Mycoplasma flocculare*

Mycoplasma hyopneumoniae e *Mycoplasma flocculare* são espécies bacterianas frequentemente encontradas no trato respiratório suíno (Petersen et al., 2016). Ambas as espécies pertencem à classe Mollicutes, no ramo denominado SEM, que abrange os gêneros *Spiroplasma*, *Entomoplasma*, *Mesoplasma*, *Mycoplasma* e *Ureaplasma* (Razin et al., 1998). A classe Mollicutes possui características diferentes de outras classes de bactérias, como ausência de parede celular, genomas ricos em AT (conteúdos de AT entre 70-80%) e tamanho reduzido (menores que 1 Mb, para a maioria) (Sirand-Pugnet et al., 2007).

O resultado da presença de *M. hyopneumoniae* e *M. flocculare* no suíno difere para cada uma delas, pois *M. hyopneumoniae* é o agente etiológico da pneumonia enzoótica suína (PES), enquanto *M. flocculare* é uma bactéria comensal (Friis, 1974). Apesar da natureza patogênica de *M. hyopneumoniae*, existem linhagens que variam quanto a seus níveis de virulência, podendo ser até mesmo avirulentas, como *M. hyopneumoniae* J (ATCC 25934). Os genomas de algumas linhagens patogênicas (232, 7448, 7422 e 168) e de uma não patogênica (J) de *M. hyopneumoniae*, assim como o genoma de *M. flocculare* já estão disponíveis para estudos comparativos (Liu et al., 2011, Minion et al., 2004, Vasconcelos et al., 2005, Siqueira et al., 2013). Os genomas reduzidos de *M. hyopneumoniae* e *M. flocculare* revelaram um número igualmente reduzido de sequências de DNA codificadoras (CDSs), resultando em um número limitado de vias metabólicas e ausências de rotas importantes, como o ciclo do ácido tricarboxílico ou síntese de aminoácidos (Rottem, 2003). Desse modo, ambas espécies são dependentes do microambiente do hospedeiro para suprir um amplo espectro de percursores necessários para a biossíntese de macromoléculas.

Devido à ausência de vias metabólicas, o meio de cultivo *in vitro* de *M. hyopneumoniae* e *M. flocculare* deve fornecer uma ampla gama de componentes necessários para o a sobrevivência e multiplicação das bactérias (Cook et al., 2016). Até os dias de hoje, o meio descrito por Friis (Friis, 1971) é o mais comumente usado para o cultivo de micoplasmas e precisa ser suplementado com soro animal (suíno, bovino ou equino). O meio de Friis também é composto por componentes biológicos complexos como extrato de levedura, caldo BHI (do inglês, *brain heart infusion*) e PPLO (do inglês, *pleuropneumonia like organism enrichment*), que podem causar variações entre as partidas dos meios de cultura e afetar a multiplicação das bactérias.

A quantificação de *M. hyopneumoniae* e *M. flocculare* continua a ser um desafio. A quantificação por contagem de colônias (CFU, do inglês *colony formation unit*), comumente aplicada para quantificação de bactérias, é imprecisa para *M. hyopneumoniae* e *M. flocculare,* pois subestima o número de células vivas e não permite que uma curva de crescimento seja feita para todos os isolados (Garcia-Morante et al., 2018). Além disso, o meio de Friis gelificado com ágar pode inibir a multiplicação e/ou sequestrar os nutrientes essenciais para a multiplicação de *M. hyopneumoniae* e *M. flocculare* (Cook et al., 2016). A quantificação baseada na unidade trocadora de cor (CCU, do inglês, *changing color units*) é considerado o "padrão-ouro", e consiste na maior diluição onde ocorreu mudança de cor devido a variação de pH pela acidificação, como resultado do metabolismo celular durante a multiplicação das bactérias (Stemke and Robertson, 1990). O número de células, no entanto, não pode ser predito através desta quantificação, mas estima-se que a maior diluição represente de 10 a 100 células de micoplasma (Loens et al., 2002). Além disso, variações podem ocorrer na reprodutibilidade do método e a lenta multiplicação de *M. hyopneumoniae* torna a quantificação baseada em CCU demorada (levando em torno de 2 semanas). Os mesmos resultados de quantificação baseados em CCU têm sido alcançados com outras técnicas, como a luminometria por ATP, a citometria de fluxo e a quantificação de DNA (Garcia-Morante et al., 2018). No entanto, ainda não há um consenso sobre o melhor método a ser utilizado e um protocolo de quantificação padronizado ainda não está vigente.

1.2. Estudos comparativos entre *M. hyopneumoniae* e *M. flocculare*

As análises comparativas realizadas entre linhagens patogênicas e não patogênicas de *M. hyopneumoniae* e entre *M. hyopneumoniae* e *M. flocculare*, até o momento, não conseguiram explicar completamente as diferenças de virulência e patogenicidade entre estas linhagens e espécies. Comparações entre os genomas das linhagens 7448 (patogênica) e J (não patogênica) de *M. hyopneumoniae* não mostrou diferenças genômicas que explicassem a diferença de patogenicidade entre estas duas linhagens (Vasconcelos et al., 2005). As análises comparativas genômicas entre *M. hyopneumoniae* e *M. flocculare* mostrou que ambas compartilham aproximadamente 78% de suas CDSs e mais de 90% das proteínas de superfície preditas (Siqueira et al., 2013). Com exceção de uma cópia do gene codificador para adesina P97, *M. flocculare* possui todo o repertório de fatores de virulência descritos em *M. hyopneumoniae*, com níveis de identidade superiores a 80% entre as proteínas ortólogas (Siqueira et al., 2013). Por outro lado,

apesar da alta identidade, 85% das proteínas de superfície compartilhadas entre estas duas espécies mostraram a presença de domínios diferenciais (Leal et al., 2016). A importância destes domínios para a diferença de patogenicidade entre estas duas espécies ainda não está clara, mas foi demonstrado que estas regiões são capazes de induzir uma resposta imune celular diferencial em camundongos.

A expressão diferencial de genes ortólogos pode desempenhar papel importante na diferença de patogenicidade e virulência entre cepas de *M. hyopneumoniae* e entre *M. hyopneumoniae* e *M. flocculare*. Estudos transcritômicos comparativos entre *M. hyopneumoniae* e *M. flocculare*, contudo, encontraram poucas diferenças no nível de transcrição de genes ortólogos codificadores de proteínas relacionadas com patogenicidade (Siqueira et al., 2014). Estudos proteômicos, em contrapartida, vêm apresentando resultados mais promissores neste sentido. Diferenças na abundância de proteínas e processamento pós-traducional diferencial foram encontrados entre cepas patogênicas e não patogênicas de *M. hyopneumoniae* (Pinto et al., 2009a). Além disso, foi demonstrado recentemente que há diferenças significativas entre os produtos de secreção solúveis de *M. hyopneumoniae* e de *M. flocculare*, incluindo potenciais fatores de virulência secretáveis (Paes et al., 2016).

A análise comparativa dos proteomas totais entre *M. hyopneumoniae* 7448, *M. hyopneumoniae* J e *M. flocculare* confirmou que fatores de virulências de *M. hyopneumoniae* 7448 de fato possuem abundância diferencial tanto na análise de frações citoplasmáticas como frações com proteínas de superfície (Paes et al., 2018). Entre estes fatores estão adesinas, proteases, transportadores de membrana e proteínas com funções *moonlighting*. Processos biológicos como adesão celular, regulação do metabolismo, detoxificação e tráfego patógeno-hospedeiro foram atribuídos a estas proteínas através de análises funcionais *in silico*.

Estudos metabólicos comparativos entre *M. hyopneumoniae* e *M. flocculare* também mostraram diferenças que podem estar relacionados com a virulência de *M. hyopneumoniae* (Ferrarini et al., 2016, Ferrarini et al., 2018). O catabolismo de mio-inositol e a produção de produtos tóxicos de peróxido de hidrogênio a partir do metabolismo de glicerol são encontrados exclusivamente em linhagens patogênicas de *M. hyopneumoniae*. No entanto, todos estes estudos proteômicos e metabólicos foram realizados com o cultivo *in vitro* das linhagens de micoplasmas sem a presença do hospedeiro.

1.3. Pneumonia enzoótica suína

A PES é uma doença crônica caracterizada por tosse esporádica e seca, febre leve e redução da conversão alimentar dos suínos, acarretando perda de peso nos animais. A distribuição geográfica das infecções com *M. hyopneumoniae* é assumida como global (Maes et al., 2017). Dados específicos da prevalência de *M. hyopneumoniae* no país não estão disponíveis na literatura, pois a notificação da doença não é considerada obrigatória. No entanto, estima-se que 95% das granjas comerciais em todo território brasileiro sejam positivas para *M. hyopneumoniae* (Barcellos et al., 2017). Em um estudo de metagenômica realizado com suínos da região Sul do Brasil, foi demonstrada a alta prevalência de *M. hyopneumoniae* tanto em pulmões de suínos saudáveis como naqueles que apresentavam lesões pulmonares (Siqueira et al., 2017).

A infecção com *M. hyopneumoniae* aumenta a suscetibilidade do suíno a coinfecções com vírus e outras bactérias, como por exemplo, *Pasteurella multocida* e o vírus da síndrome suína reprodutiva e respiratória (PRRSV, do inglês *porcine reproductive and respiratory syndrome virus*), contribuindo para o desenvolvimento do chamado complexo de doenças respiratórias suínas (CDRS) (Thacker and Minion, 2010). Além disso, a presença de *M. hyopneumoniae* predispõe os animais a uma reação inflamatória exacerbada em resposta à infecção com outros patógenos, o que pode aumentar a severidade das lesões pulmonares resultantes das coinfecções (Deblanc et al., 2016, Maes et al., 2017). As consequências das coinfecções variam de acordo com os patógenos envolvidos e ainda não está claro como estes patógenos interagem com *M. hyopneumoniae* no trato respiratório suíno.

A PES é altamente contagiosa, pois *M. hyopneumoniae* é capaz de se disseminar rapidamente sob condições ambientais favoráveis em suínos na fase de crescimento e terminação (Maes et al., 2017). A transmissão de *M. hyopneumoniae* ocorre principalmente por contato direto através das secreções nasais e aerossóis eliminados durante os episódios de tosse e espirros, via vertical, da porca para os leitões, ou via horizontal, de leitão para leitão (Garza-Moreno et al., 2018). Práticas inadequadas de manejo, incluindo alta densidade dos animais, falta de higiene das instalações e fatores ambientais, podem aumentar a concentração de contaminantes aéreos, o que potencializa os impactos da PES (Sibila et al., 2009).

O isolamento de *M. hyopneumoniae* dos pulmões de suínos por cultivo bacteriológico é considerado a técnica padrão-ouro para o diagnóstico (Thacker, 2004). A necessidade de um meio especializado, a lenta multiplicação do organismo e a contaminação por outros micoplasmas, como

M. flocculare e *M. hyorhinis* impede a utilização da técnica rotineiramente. A aplicação da técnica de PCR tem permitido o aumento na sensibilidade de detecção de *M. hyopneumoniae*, e, hoje em dia, constitui-se em um dos métodos mais comuns para a detecção de *M. hyopneumoniae* em diferentes tipos de amostra, como fluidos orais e swabs nasais (Maes et al., 2017). Kits comerciais sorológicos de ELISA também estão disponíveis para detecção de anticorpos contra *M. hyopneumoniae* (Sibila et al., 2009, Maes et al., 2017). Estes testes comerciais se baseiam em diferentes antígenos e plataformas de ELISA, mas apresentam acurácias similares e são incapazes de diferenciar suínos que tenham sido vacinados daqueles que sofrem da doença. Além disso, na fase crônica da infecção, os anticorpos contra *M. hyopneumoniae* diminuem e são dificilmente detectados.

A vacinação é uma das estratégias mais comumente usadas para o controle das infecções com *M. hyopneumoniae* no campo, e possui uma série de benefícios, como por exemplo, a redução da pressão de infecção nos pulmões, diminuição dos sinais clínicos e lesões pulmonares, e o aumento do ganho de peso diário (Barcellos et al., 2017, Pieters and Sibila, 2017). As vacinas comerciais disponíveis são células de *M. hyopneumoniae* inteiras inativadas, chamadas bacterinas. No entanto, a vacinação não impede a transmissão de *M. hyopneumoniae* entre os animais (Meyns et al., 2006), a adesão da bactéria às células suínas e a colonização do trato respiratório suíno pela bactéria (Villarreal et al., 2012). Devido aos problemas enfrentados com vacinas baseadas em bacterinas de *M. hyopneumoniae*, estudos com antígenos recombinantes têm demonstrado potencial (Conceição et al., 2006, Simionatto et al., 2013, Galli et al., 2012, Virginio et al., 2014). No entanto, na maioria destes estudos a resposta imune foi avaliada somente em camundongos e apenas algumas poucas formulações vacinais recombinantes foram testadas em suínos. Algumas das vacinas testadas em suínos, baseadas em subunidades recombinantes das adesinas P97 ou P42, foram capazes de induzir a produção de imunoglobulinas IgG e IgA, mas não conferiram proteção significativa contra infecção com *M. hyopneumoniae* (Marchioro et al., 2014). A falta de proteção da vacina demonstra que mais estudos são necessários para melhorar a eficiência das vacinas baseadas em subunidades. Além disso, a validação do uso destas vacinas em circunstâncias práticas nos rebanhos suínos ainda é necessária.

1.4. Respostas do hospedeiro à infecção com *M. hyopneumoniae*

M. hyopneumoniae se adere externamente às células do epitélio ciliar do trato respiratório de suínos (Young et al., 2000). A adesão depende de adesinas presentes na superfície de *M. hyopneumoniae*. As adesinas medeiam o processo de interação com proteínas da superfície ciliar e da matriz extracelular do hospedeiro. Diversas moléculas do hospedeiro que interagem com *M. hyopneumoniae* já foram identificadas, como, por exemplo, actina extracelular, heparina, fibronectina e plasmonigênio (Raymond et al., 2018, Seymour et al., 2011, Raymond et al., 2014, Seymour et al., 2012), mas o papel destas moléculas durante a infecção ainda não está totalmente elucidado.

A infecção por *M. hyopneumoniae* induz hiperplasia das células epiteliais, a presença de leucócitos e macrófagos nos septos, brônquios e bronquíolos (Pósa et al., 2014). As proteínas da superfície celular de *M. hyopneumoniae* interagem com plasminogênio disponível nas vias aéreas do suíno (Seymour et al., 2011), ativando sua conversão em plasmina (Seymour et al., 2012). Um vez ativada, a plasmina estimula a sinalização intracelular nas células suínas via fatores de transcrição MAPK e NF-kB, o que resulta na liberação de citocinas pró-inflamatórias (Damte et al., 2011, Hwang et al., 2011). O aumento na quantidade de plasmina nas vias áreas do suíno esta positivamente relacionado com a produção das TNF-α e IL-1β (Woolley et al., 2013). Além da grande quantidade de células, a produção excessiva das citocinas pró-inflamatórias também acarreta dano ao tecido epitelial do hospedeiro. Desse modo, a resposta imune do suíno é um componente importante do quadro patológico da PES, sendo considerada o principal determinante do desenvolvimento das lesões pulmonares observadas (Damte et al., 2011).

M. hyopneumoniae demonstra a capacidade de modular a resposta imune do suíno devido ao caráter crônico da PES. O mecanismo exato desta modulação ainda não está totalmente elucidado, mas foi demonstrado que *M. hyopneumoniae* pode suprimir a resposta imune humoral e celular, pois reduz a capacidade dos linfócitos de produzir anticorpos e inibe a fagocitose mediada por macrófagos (Maes et al., 1996). Essa supressão é mais pronunciada nos primeiros estágios pós-infecção, mas pode durar várias semanas após o início da infecção. Além disso, após 28 dias de infecção com *M. hyopneumoniae*, ocorre a diminuição do número de células dendríticas na cavidade nasal dos suínos e a redução da capacidade destas células de estimularem células T do sistema imune celular (Shen et al., 2017).

M. hyopneumoniae também possui a capacidade de induzir apoptose em células suínas. Alguns estudos demonstraram que proteínas da superfície de *M. hyopneumoniae* são capazes de induzir apoptose em macrófagos alveolares, células sanguíneas mononucleares e células epiteliais de suínos (Bai et al., 2013, Bai et al., 2015, Paes et al., 2017b). Um dos mecanismos de apoptose conhecidos é o mediado pela liberação do citocromo C no citoplasma resultando na ativação das caspases 3 e 8 (Ni et al., 2015, Bai et al., 2015). A proteína p38 (MAPK, do inglês *mitogen-activated protein kinase*), outra proteína da via das caspases para indução da apoptose, é fosforilada e ocorre um aumento na expressão das proteínas pró-apoptóticas Bax, enquanto a expressão da proteína anti-apoptótica Bcl-2 é diminuída. A ativação das vias das caspases tem sido associada à produção de espécies reativas de oxigênio, como óxido nítrico e ânion superóxido, visto que a inibição das ROS pelo uso de antioxidantes diminui a ativação de caspase-3 e a taxa de apoptose em macrófagos *in vitro* (Bai et al., 2013).

2. JUSTIFICATIVAS

Segundo a Associação Brasileira dos Criadores de Suínos (ABCS, http://www.abcs.org.br/), a carne suína é uma das fontes de proteína animal mais consumidas no mundo, sendo a suinocultura uma atividade importante para economia brasileira, principalmente da região Sul. Infelizmente, a cadeia produtiva de suínos brasileira é significativamente impactada por doenças infecciosas respiratórias, como a PES (Chae, 2016). As dificuldades para o diagnóstico da infecção por *M. hyopneumoniae* e para a vacinação contra a doença decorrem principalmente dos conhecimentos ainda limitados da fisiologia do *M. hyopneumoniae* e de suas interações com o suíno (Maes et al., 2017). A comparação de extratos celulares totais de linhagens patogênicas e não patogênicas de *M. hyopneumoniae* (Pinto et al., 2009b, Paes et al., 2018), assim como seus secretomas (Paes et al., 2017a), demonstraram que abordagens proteômicas prospectivas e comparativas são viáveis para *M. hyopneumoniae* e permitem identificar moléculas potencialmente relevantes para a patogenicidade da bactéria. No entanto, todos estes estudos foram conduzidos em condições experimentais nas quais o hospedeiro era representada única e exclusivamente pela presença de soro suíno no meio de cultivo.

Estudos proteômicos de interação patógeno-hospedeiro envolvendo situações de infecção natural ou experimental do hospedeiro suíno ainda não são viáveis, em função da dificuldade e dos custos de manutenção de suínos livres de patógenos e a impossibilidade da técnica de detectar e

quantificar de forma confiável proteínas secretadas em amostras de tecido pulmonar suíno. O uso de linhagens celulares para estudos de interações patógeno-hospedeiro em alternativa à experimentação animal vem sendo aplicado para os mais diversos organismos (Li et al., 2014, Lietzén et al., 2011). A análise *in vitro* das proteínas envolvidas durante a infecção das células suínas com as linhagens de micoplasmas permite condições de interações controladas com apenas uma ou outra espécie, e identificação de respostas específicas contra cada espécie.

A linhagem celular NPTr, uma linhagem celular isolada de células da traqueia de um suíno (Ferrari et al., 2003), irá mimetizar o tipo celular ou o ambiente em que as linhagens de micoplasmas estão durante a infecção. Desse modo, processos locais celulares poderão ser elucidados na interação entre o hospedeiro e *M. hyopneumoniae*. A comparação das respostas das linhagens celulares frente a cada micoplasma e de cada micoplasma às células hospedeiras podem elucidar mecanismos responsáveis pela diferença de patogenicidade entre as duas linhagens ou espécies. Sua realização em moldes comparativos, com *M. hyopneumoniae* e *M. flocculare*, permitirá, além da identificação de diferenças na resposta de células suínas a cada espécie, a identificação de novos determinantes do hospedeiro para o quadro clínico da PES.

Até o momento, nenhum estudo proteômico foi realizado para a identificação de proteínas envolvidas na relação patógeno-hospedeiros durante o processo de patogenicidade de *M. hyopneumoniae*. Estudos comparativos ao nível proteico podem fornecer mais evidências das interações patógeno-hospedeiro, quando comparados com estudos genômicos e transcriptômicos, visto que a abundância proteica será o resultado da regulação transcricional, processamento pós-traducional e/ou degradação proteica (Paes et al., 2018). A identificação de proteínas intracelulares, proteínas totais secretadas e proteínas secretadas por vesículas extracelulares de uma linhagem de células suínas em resposta a diferentes espécies e cepas de micoplasmas, irá permitir uma análise global das mudanças celulares durante à infecção. A identificação de proteínas alteradas apenas durante a infecção com o *M. hyopneumoniae* patogênico deverão evidenciar proteínas potencialmente relevantes como determinantes de patogenicidade de *M. hyopneumoniae*. A descoberta dos determinantes suínos de patogenicidade de *M. hyopneumoniae* poderão auxiliar no desenvolvimento de novos tratamentos e na prevenção da PES.

3. OBJETIVOS

3.1 Objetivo geral

O objetivo geral deste trabalho foi identificar e comparar as proteínas diferencialmente representadas durante a infecção de uma linhagem celular suína com micoplasmas patogênicos e não patogênicos do trato respiratório suíno para a identificação de vias de resposta de células suínas à infecção com *M. hyopneumoniae* e determinantes suínos do quadro clínico da pneumonia enzoótica suína.

3.2 Objetivos específicos

i) Padronizar ensaios de infecção entre uma linhagem celular suína e linhagens de *M. hyopneumoniae* e *M. flocculare*;
ii) Identificar proteínas secretadas diferencialmente representadas em situações de infecção de células suínas com *M. hyopneumoniae* ou *M. flocculare*;
iii) Identificar proteínas intracelulares suínas diferencialmente representadas durante a infecção com *M. hyopneumoniae* ou *M. flocculare*;
iv) Identificar proteínas secretadas via vesículas extracelulares em situações de infecção de células suínas com *M. hyopneumoniae*;
v) Evidenciar determinantes suínos da PES e vias de resposta de células suínas à infecção com *M. hyopneumoniae*.

4. MATERIAIS E MÉTODOS, RESULTADOS E DISCUSSÃO

Na seção 4.1, métodos, resultados e discussão referentes à padronização do ensaio de infecção entre a linhagem celular suína NPTr e *M. hyopneumoniae* 7448, *M. hyopneumoniae* J ou *M. flocculare* e identificação das proteínas diferencialmente representadas no sobrenadante dos ensaios de infecção das células NPTr com *M. hyopneumoniae* 7448, *M. hyopneumoniae* J ou *M. flocculare*, são apresentados na forma de artigo publicado na revista *Journal of Proteomics*.

Na seção 4.2, métodos, resultados e discussão referentes à identificação das proteínas intracelulares diferencialmente representadas durante a infecção das células NPTr com *M. hyopneumoniae* 7448, *M. hyopneumoniae* J ou *M. flocculare*, são apresentados na forma de manuscrito a ser submetido na revista *Frontiers in Cellular and Infection Microbiology*.

Na seção 4.3, métodos e resultados referentes à identificação das proteínas secretadas por vesículas extracelulares diferencialmente representadas durante a infecção das células NPTr com *M. hyopneumoniae* 7448.

O Material Suplementar citado nas seções de 4.1 a 4.3 está disponível no CD anexado nesta tese.

4.1 Perfil de secretoma diferencial de uma linhagem celular de traqueia suína infectada com micoplasmas do trato respiratório suíno.

Manuscrito publicado no periódico *Journal of Proteomics*

Differential secretome profiling of a swine tracheal cell line infected with mycoplasmas of the swine respiratory tract

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Contribuição dos autores:

F.M.A.L.Z: delineamento experimental, execução de todos os experimentos e redação do manuscrito. G.P.P: construção e análise das redes de co-expressão; H.M e J.R.B.: auxílio nos experimentos de proteômica *bottom up* e revisão do manuscrito; H.B.F: delineamento experimental, análise e discussão dos resultados e revisão do manuscrito.

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Differential secretome profiling of a swine tracheal cell line infected with mycoplasmas of the swine respiratory tract

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ABSTRACT

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* are genetically similar. However, *M. hyopneumoniae* causes porcine enzootic pneumonia, while *M. flocculare* is a commensal bacterium. *M. hyopneumoniae* and *M. flocculare* do not penetrate their host cells, and secreted proteins are important for bacterium-host interplay. Thus, the secretomes of a swine trachea cell line (NPTr) infected with *M. hyopneumoniae* 7448 (a pathogenic strain), *M. hyopneumoniae* J (a non-pathogenic strain) and *M. flocculare* were compared to shed light in bacterium-host interactions. Medium from the cultures was collected, and secreted proteins were identified by a LC-MS/MS. Overall numbers of identified host and bacterial proteins were, respectively, 488 and 58, for NPTr/*M. hyopneumoniae* 7448; 371 and 67, for NPTr/*M. hyopneumoniae* J; and 203 and 81, for NPTr/*M. flocculare*. The swine cells revealed different secretion profiles in response to the infection with each *M. hyopneumoniae* strain or with *M. flocculare*. DAMPs and extracellular proteasome proteins, secreted in response to cell injury and death, were secreted by NPTr cells infected with *M. hyopneumoniae* 7448. All three mycoplasmas secreted virulence factors during NPTr infection, but *M. hyopneumoniae* 7448 secreted higher number of adhesins and hypothetical proteins, that may be related with pathogenicity.

Significance: The enzootic pneumonia caused by mycoplasmas of swine respiratory tract has economic loss consequences in pig industry due to antibiotic costs and pig weight loss. However, some genetically similar mycoplasmas are pathogenic while others, such as *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*, are non-pathogenic. Here, we conducted an infection assay between swine cells and pathogenic and non-pathogenic mycoplasmas to decipher secreted proteins during host-pathogen interaction. Mycoplasma response to cell infection was also observed. Our study provided new insights on secretion profile of swine cells in response to the infection with pathogenic and non-pathogenic mycoplasmas. It was possible to observe that pathogenic *M. hyopneumoniae* 7448 secreted known virulence factors and swine cells responded by inducing cell death. Otherwise, *M. hyopneumoniae* J and *M. flocculare*, non-pathogenic mycoplasmas, secreted a different profile of virulence factors in response to swine cells. Consequently, swine cells altered their secretome profile, but the changes were not sufficient to cause disease.

1. Introduction

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* cohabit the swine respiratory tract [1]. Both are genetically very similar, and share around 78% of CDSs and 90% of predicted surface proteins, including all known virulence factors [2]. Only minor expression differences between ortholog genes coding for proteins involved with pathogenicity were detected at the transcriptional level [3]. However, despite of their overall similarities, *M. hyopneumoniae* is the etiological agent of porcine enzootic pneumonia (PEP), a chronic respiratory disease, that causes significant economic losses to the commercial swine production industry, while *M. flocculare* is a commensal bacterium. Moreover, there are virulence variations among strains of *M. hyopneumoniae*, ranging from completely avirulent strains (e.g. *M. hyopneumoniae* J) to highly

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virulent ones (e.g. *M. hyopneumoniae* 7448) [4,5].

M. hyopneumoniae is an extracellular bacterium, and adherence to the host epithelium, mediated by adhesins, is a required step to establish infection [6,7]. The ability of adhesins to strongly bind to host molecules such as glycosaminoglycans, heparin, fibronectin and plasminogen is important for the early stages of colonization [8–12]. Upon adherence, *M. hyopneumoniae* causes damage to epithelial ciliary cells of trachea, bronchi and bronchioles and cell death [13]. *M. hyopneumoniae* cytotoxicity has been associated to different factors, from blocking of Ca^{2+} channels [14] to specific surface proteins, like lipid-associate membrane proteins (LAMPs) [15], or signal peptidase I [16]. Comprehensive comparative surveys between *M. hyopneumoniae* strains differing in virulence, have so far, contributed to define bacterial PEP determinants. For instance, differences between pathogenic and non-pathogenic strains have been found at the proteome level, including differential abundances and/or differential proteolytic processing of adhesins and other surface proteins [17,18].

M. flocculare, on the other hand, is also capable of adhering to epithelial ciliary cells and colonizing the swine respiratory tract, but no damage is observed resulting of its presence [19]. Differences between *M. hyopneumoniae* and *M. flocculare* have been found in recent years, such as differential domains among orthologous surface proteins [20], and distinct repertoires of secreted proteins [21]. The differences identified between *M. hyopneumoniae* strains and between *M. hyopneumoniae* and *M. flocculare* point to a complex scenario of multifactorial virulence determinants in swine mycoplasmas, which demands further studies to explain the differences in pathogenicity and virulence.

Swine host response to *M. hyopneumoniae* infection involves acute inflammation of the respiratory tract, with epithelial hyperplasia and infiltration of neutrophils, mononuclear and inflammatory cells from lamina propria [22]. Alveolar macrophages and lymphocytes, stimulated by *M. hyopneumoniae*, produce pro-inflammatory cytokines such as interleukin (IL)-1β, and IL-18 [23–27] that determine lung lesions and lymphoid hyperplasia [28]. Due to the importance of host response to *M. hyopneumoniae* pathogenesis, and the fact that this species does not penetrate the swine cells, proteins secreted by both swine and bacterium are most likely be important for infection outcome. Comparisons between the response of swine cells to pathogenic and non-pathogenic *M. hyopneumoniae* strains, as well as between *M. hyopneumoniae* and *M. flocculare,* are expected to help explaining why such closely related strains and species differ in pathogenicity.

The present study was performed to compare the secreted protein profile of swine cells infected with the *M. hyopneumoniae* 7448 pathogenic strain with those of the same swine cells infected with the non-pathogenic *M. hyopneumoniae* J strain or the commensal *M. flocculare*. These mycoplasma strains and species were used to infect porcine NPTr cells, an epithelial trachea cell line, in order to identify proteins secreted by both swine and bacterium using a detailed liquid chromatography (LC)-tandem mass spectrometry (MS/MS) proteomic approach. Profiles of secretory pathway prediction, functional analyses of swine proteins and identification of mycoplasma virulence factors differentially presented during infection were found to each *M. hyopneumoniae* strain or *M. flocculare*. The implications of differential secretome profiling to each mycoplasma and their role in PEP will be discussed.

2. Materials and methods

2.1. M. hyopneumoniae and M. flocculare cultures

M. hyopneumoniae 7448 was originally isolated from a naturally infected pig from Lindóia do Sul (SC, Brazil) and its pathogenicity was experimentally demonstrated [5]. The non-pathogenic *M. hyopneumoniae* J (ATCC 25934), and the commensal *M. flocculare* (ATCC 27716) were originally acquired by Empresa Brasileira de Pesquisa Agropecuária-Centro Nacional de Pesquisa de Suínos e Aves (EMBRAPA-CNPSA, Concórdia, SC, Brazil) and kindly donated to our research group. *M. hyopneumoniae* J has a reduced adhesion capacity to the porcine respiratory epithelium [5], and *M. flocculare* is capable of adhere to respiratory epithelial cells, but does not cause disease [19]. *M. hyopneumoniae* strains and *M. flocculare* were separately cultured in Friis medium [29] containing 25% of fetal bovine serum (FBS) (Gibco™, Thermo Fisher Scientific, IL, USA), in 25 ml batches, at 37 °C for 48 h with agitation, as previously described [20]. After culture, mycoplasma cells were centrifuged at 3500*g* for 15 min, washed three times with PBS pH 7.4, for medium removal, and resuspended in 5 ml of Minimal Essential Medium with Earle's Balanced Salt Solution and L-glutamine (MEM/EBSS; LGC Biotecnologia, SP, Brazil), prior to use in infection assays.

2.2. Porcine cell cultures

Newborn Porcine Trachea (NPTr) cells [30] were purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy), and cultured in MEM/EBSS supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. Approximately 5×10^5 cells were seeded in 75 cm^3 plates and grown for 48 h until 60–70% confluence. After 48 h, cells were washed five times with serum free-MEM/EBSS (serum-free medium, SFM) to remove FBS contaminants. MEM/EBSS SFM was added and cells were cultured for 12 h, 18 h, or 24 h. Cell growth and viability were measured with trypan blue exclusion test and by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), respectively.

2.3. Infection assays and sample preparation for LC-MS/MS analyses

NPTr cells were initially cultured in MEM/EBSS SFM as described above. After suspension in SFM, *M. hyopneumoniae* 7448, *M. hyopneumoniae* J or *M. flocculare* 5 ml-batches (10^2 CCU/ml) were added, and co-cultures were additionally incubated for 18 h at 37 °C in a humidified atmosphere containing 5% CO_2. Cultures of NPTr cells without mycoplasma infection, and *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare* cultures in SFM without swine cells were used as controls. Control cultures were carried out in the same conditions described above for the infection assays.

Each infection assay, and each control culture was independently performed three times (biological replicates). Medium from each culture (~15 ml) was recovered, and samples were prepared for mass spectrometry as described by [31], with some modifications. Briefly, supernatants of 18 h-cultures were centrifugated twice, first at 3500 g for 15 min, to pellet mycoplasma cells, and then, at 9000 g for 15 min, to pellet swine cells and cellular debris. Recovered culture supernatants were filtered through a 0.22 μm filter (Chemicon, SP, Brazil), and concentrated using Amicon Ultra-15 (Millipore, MA, USA) centrifugal filter devices with a 3000-nominal molecular weight limit. Each concentrated supernatant (~1 ml) was divided into two equal aliquots, and proteins were subsequently precipitated with 5 volumes of acetone at −20 °C overnight. Precipitated proteins in each aliquot were harvested by centrifugation at 16,000*g* for 20 min, and the resulting protein pellets were dried in a Concentrator Plus (Eppendorf, DE). For each experimental or control sample, one aliquot was resuspended in 0.3 ml of PBS (pH 7.4), and used for initial qualitative and quantitative evaluation of the protein content, while the other, was solubilized in 0.3 ml of 8 M urea, and further processed for analysis by LC-MS/MS. Quantitation was performed using the microBCA Protein Assay Kit (Thermo Fisher Scientific, IL, USA), and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, IL, USA). For LC-MS/MS, 40 μg of protein in 8 M urea were reduced with 2 μg of dithiothreitol (Thermo Fisher Scientific, IL, USA) at 37 °C for 1 h, and alkylated with 10 μg of iodoacetamide (Bio-Rad, CA, USA) in the dark at room temperature for 30 min. Protein samples were diluted with 25 mM ammonium bicarbonate (pH 8.0) to a final 1 M urea concentration, and further digested with

1 μg of trypsin (Promega, WI, USA). Resulting peptides were desalted in HLB cartridges (Waters Corporation, MA, USA) and eluted with 50% acetonitrile/0.1% trifluoracetic acid (TFA). Peptides were then dried using a Concentrator Plus (Eppendorf, DE), and kept at −20 °C, until analysis.

2.4. SDS-PAGE and western-blotting

Quantitative and qualitative assessment of protein samples in culture supernatants was carried out by SDS-PAGE and western-blotting. SDS-PAGE was performed using NuPAGE™ 4–12% Bis-Tris gels (Invitrogen, CA, USA). Samples (10 μl) of concentrated protein aliquots in PBS were resolved and stained with Pierce® Silver Stain for Mass spectrometry (Thermo Fisher Scientific, IL, USA), according to the manufacturers' instructions.

The analyzed culture supernatants (30 μg of pool samples) were assessed for the presence of swine intracellular protein markers by western-blotting using an iBlot® Dry Blotting System (Thermo Fisher Scientific, IL, USA), according to the manufacturer's instructions. A monoclonal anti-β-actin antibody (Sigma-Aldrich, MO, USA), and polyclonal antibodies anti-calnexin (Sigma-Aldrich, MO, USA) were used as primary antibodies. NPTr cell total protein extract (30 μg) was used as positive control for both antibodies. Blocking and detection were performed using WesternBreeze® Chromogenic Immunodetection System (Thermo Fisher Scientific, IL, USA), according to the manufacturer's instructions.

2.5. LC-MS/MS for protein identification

Peptide samples from culture supernatants (section 2.3) were reconstituted in a 0.1% formic acid (FA) in water solution (Thermo Fisher Scientific, IL, USA), and applied to an on-line LC-MS/MS system, including a HPLC NanoAcquity System (Waters Corporation, MA, USA), and an Orbitrap Elite™ Hybrid IonTrap-Orbitrap mass spectrometer (Thermo Fischer Scientific, IL, USA), as follows. A 5 μl volume of each sample (corresponding to 5 μg of tryptic peptides) was loaded onto a PepMap® 100 C18 LC Column (0.3 mm × 5 mm) (Thermo Fischer Scientific, IL, USA) at a flow rate of 5 μl/min. Peptides were eluted to an Easy-Spray Column PepMap® RSLC C18 (75 μm × 15 cm) (Thermo Fischer Scientific, IL, USA) using an HPLC gradient formed by HPLC-grade water with 0.1% FA (solvent A), and 0.1% FA in acetonitrile solution (solvent B, Thermo Fisher Scientific, IL, USA). The gradient flow was set at 0.3 μl/min, and its profile consisted of a hold at 5% solvent B for 5 min, a ramp up to 35% solvent B over 65 min, a ramp up to 95% solvent B in 5 min, and a hold at 95% for 5 min, prior to returning to 5% solvent B in 5 min, and re-equilibration at 5% solvent B for 20 min. After the chromatography steps, the peptides were introduced into the mass spectrometer. 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 range from mass-to-charge (m/z) 400 to 1600 at a nominal resolution setting of 60,000 for parent ion acquisition. For the LC-MS/MS analyses, the mass spectrometer was programmed to select the top 15 most intense ions with two or more charges. The resolution for the fragment ions was 0.5 a.m.u using collision-induced dissociation (CID). Dynamic exclusion was enabled with an exclusion duration of 60 s. Each biological replicate was independently analyzed by LC-MS/MS three times (technical replicates).

2.6. LC-MS/MS data analysis

MS data was processed using the Mascot Software (Matrix Science, UK). Raw MS data files were processed using Mascot Distiller. The database search was performed against local *Sus scrofa*, *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare* databases containing the deduced amino acid sequences (with 48,617; 819; 793; and 758 sequences, respectively), derived from the genome annotations available at NCBI (https://www.ncbi.nlm.nih.gov/protein). For both swine and mycoplasma protein identifications the search parameters included a fragment ion mass tolerance of 0.6 Da, peptide ion tolerance of 50 ppm. For swine proteins, one missed cleavage of trypsin was allowed, carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine was included as variable modifications. For mycoplasma proteins, three missed cleavages of trypsin were allowed, carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine, acetylation of lysine and N-terminal ends of proteins, and phosphorylation of tyrosine and serine/threonine were included as variable modifications. Additional variable modifications were included for mycoplasma searches due to the high content of adenine and thymine in mycoplasma genomes, and the consequent increase of the lysine and serine content in the encoded proteins [32,33].

Scaffold software version 4.4.1 (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 > 95.0% probability as assigned by the Peptide Prophet algorithm [34]. The protein identifications were accepted if they could be established at > 99% probability as assigned by the Protein Prophet algorithm [35]. Swine protein identifications were validated only when at least 2 corresponding peptides were detected. For mycoplasma proteins, when a single peptide was used to validate an identification, the corresponding MS/MS spectrum was manually inspected and was accepted only when it showed at least five consecutive b- or y-type fragment ions [21,36]. Any validated identified proteins were accepted only if they were detected in at least two of three biological replicates. The normalized spectral abundance factor (NSAF) [37] was calculated for each identified protein in Scaffold software and used to quantify relative differences in protein abundance between samples. Missing values were treated as zeros and incorporated into the analysis. Quantitative differences were statistically analyzed using the Student's t-test in GraphPad Prism 6 software. Proteins with a p value < .05 and a FC > 1.5 were considered differentially abundant by both statistical and FC parameters.

2.7. Bioinformatics analysis of protein function and cellular localization

Swine proteins were submitted to hierarchical GO over-representation tests using the Cytoscape 2.6.3 26 plugin BiNGO 2.3 [38]. Swine annotation files were acquired from Uniprot database (http://www.uniprot.org), and 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 and underrepresentation tests were performed at 0.001 and 0.05 levels of significance, respectively, with the Benjamini-Hochberg FDR multiple-testing correction. The 350 overrepresented subcategories with highest p values from each enriched GO term-list categories were summarized by removing redundant GO terms using REVIGO [39]. The semantic similarity of the GO terms was calculated through SimRel (default allowed similarity = 0.7), and the results were plotted using R, a system for statistical computation and graphics [40].

In silico predictions of secretion pathways for swine and mycoplasma identified proteins were performed using the pipeline described by [41]. Signal peptide-dependent secretion was predicted using SignalP 4.1 [42], Phobius [43], and PrediSI [44]. Signal peptide-independent secretion predictions were performed using SecretomeP 2.0 [45]. A protein was considered to have a signal peptide, and as secreted by the “classical pathway” if its signal peptide was predicted by at least two of the algorithms used. Proteins with no prediction of signal peptide, but with a SecretomeP NN score > 0.6 were classified as secreted by a “non-classical pathway”. Detected proteins with no prediction of signal peptide or non-classical secretion were further analyzed for

ortholog presence in extracellular vesicles by searches in Vesiclepedia [46].

2.8. Construction and analysis of protein co-expression networks

Protein co-expression networks were built for the sets of proteins identified in culture supernatants from infection assays. NSAF protein values were used as input to the WGCNA package [47]. The networks were constructed based on a Pearson correlation matrix between all proteins, and converted into a signed adjacency matrix applying a power function with a customizable power parameter, with the threshold of co-expression set as $p < .05$. Only connections with values > 0.06 were selected, and the networks were saved in a compatible format for analysis in the Cytoscape software version 3.6.0. Clusters identifications in the networks were performed using the Cytoscape clusterMaker plugin by the MCODE method, and a degree cut-off of 2 [48,49]. Cluster subnetworks were enriched with physical protein-protein interaction data obtained from the STRING database (https://string-db.org). The enrichment analyses were performed using the STRING classification systems for Gene Ontology and KEGG, applying a Fisher's exact test followed by a correction for multiple testing [50].

3. Results

3.1. NPTr cell cultures and infections with mycoplasmas

The effects of FBS removal on NPTr cell viability and proliferation were measured by MTT test and cell counting, respectively, after 12 h, 18 h and 24 h incubation in 10% FBS MEM/EBSS (control) or SFM. In the MTT assays (Fig. S1A), no significant differences in cell viability between cells grown in SFM and control cultures were observed. The proliferation assay (Fig. S1B) showed that NPTr cells cultured in SFM were proliferative, although reduced in comparison to control cultures. Maximum NPTr cell growth was observed at 18 h.

NPTr cells were infected or not (NPTr control cultures) with *M. hyopneumoniae* 7448 (NPTr-Mh7448 cultures), *M. hyopneumoniae* J (NPTr-MhJ cultures) or *M. flocculare* (NPTr-Mf cultures) for 18 h. After this time, NPTr-Mh7448, NPTr-MhJ, NPTr-Mf, and NPTr control cultures had their morphologies inspected at the microscope (Fig. 1). In comparison to the NPTr control cultures (Fig. 1A), NPTr cells from NPTr-Mh7448, NPTr-MhJ, NPTr-Mf cultures (Fig. 1B, C and D, respectively) presented reduction in confluence and presence of cell debris. Some morphology changes were more evident in NPTr-Mh7448 cultures, where most NPTr cells presented irregular shapes. Other culture conditions, as cell densities and washing protocols were tested (data not shown), with no observed improvement in the results in comparison to described above. Percentage of apoptotic cells increased ~10% in NPTr cells infected with *M. hyopneumoniae* 7448 in comparison with NPTr control cells (data not shown).

3.2. SDS-PAGE and immunoblot profiling of culture supernatants

Overall, similar SDS-PAGE profiles were observed for all replicas of NPTr-Mh7448, NPTr-MhJ, NPTr-Mf cultures, and control cultures (Fig. S2A). Immunoblotting analysis was used to investigate the presence of the 42 kDa β-actin and 67 kDa calnexin intracellular markers in supernatant samples from NPTr-Mh7448, NPTr-MhJ, NPTr-Mf, and control cultures (Fig. S2B). The β-actin band was faintly detected in the NPTr control and in the NPTr-Mh7448, NPTr-MhJ, and NPTr-Mf samples as well, but not, as expected, in the mycoplasma control cultures. The detected β-actin band was more intense in NPTr-MhJ sample, but still much less intense than in the NPTr total cell extract control. Calnexin was not detected in any of the analyzed samples, except for the positive control.

3.3. MS-based identification of swine and mycoplasma proteins from supernatant samples

Totals of 287, 488, 371 and 203 swine proteins were identified by LC-MS/MS in supernatants from NPTr control, NPTr-Mh7448, NPTr-MhJ and NPTr-Mf, respectively. Detailed protein and peptide identification and quantification data are presented in Tables S1 and S2, respectively. The false discovery rate (FDR) for the proteins and peptides of all samples was zero, validating all MS/MS results. The Venn diagram

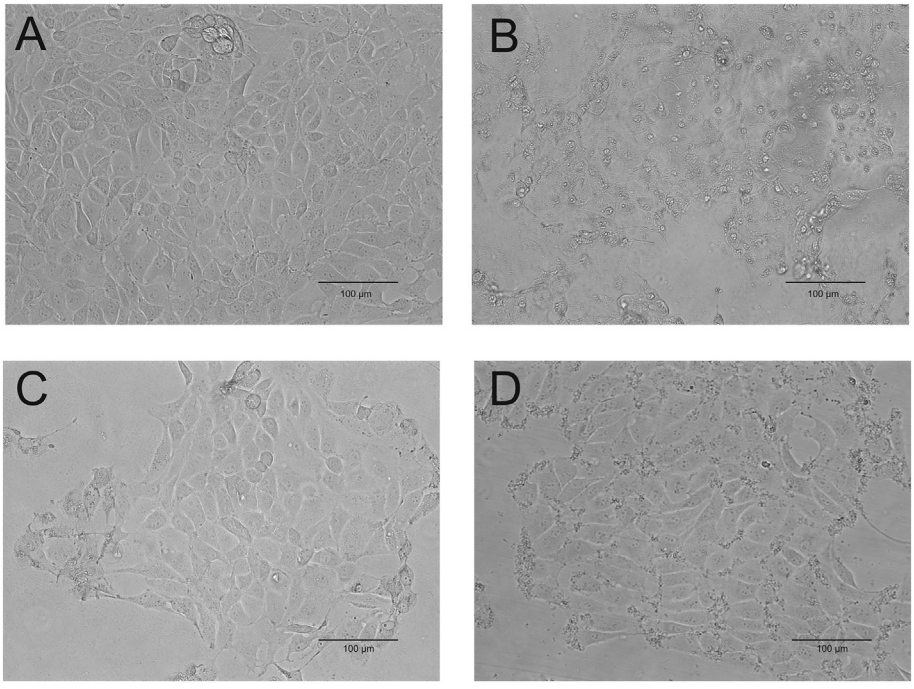

Fig. 1. NPTr cells morphology. (A) NPTr cells culture control, NPTr cells infected with (B) *M. hyopneumoniae* 7448, (C) *M. hyopneumoniae* J and, (D) *M. flocculare*. The image was obtained using EVOS FLoid (scale bar = 100 µm).

in Fig. 2A presents an overview of exclusively and shared protein in the analyzed samples. A set of 160 swine proteins was shared by all samples, including the most represented proteins, according to NSAF values (Table S1). Among these highly represented, shared proteins, are retinol-binding protein, two galectins, thymosin beta-10, 14–3-3 protein zeta/delta, peptidyl-prolyl cis-trans isomerase A, actin, alpha-enolase, vimentin, profilin, protein S100-A11, annexin A8, and transgelin-2. On the other hand, 31, 121, 24 and 1 proteins were identified exclusively in NPTr control, NPTr-Mh7448, NPTr-MhJ and NPTr-Mf samples, respectively. It is interesting to note, the high contrast between the number of exclusive proteins in the NPTr-Mh7448 secretome sample in comparison to that of the NPTr-Mf samples.

Considering that mycoplasma cultures were performed in medium containing FBS, LC-MS/MS data of supernatant samples from *M. hyopneumoniae* 7448, *M. hyopneumoniae* J and *M. flocculare* control cultures were analyzed using a swine database, to identify bovine ortholog proteins. The resulting protein and peptide identification data are presented in Tables S3 and S4, respectively. Thus, 35 different protein species were identified for the *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare* control cultures. These proteins were assumed as serum proteins, and removed from the swine protein lists, to exclude any false positive protein identifications due to residual FBS contamination.

LC-MS/MS data from NPTr-Mh7448, NPTr-MhJ and NPTr-Mf supernatants, along with LC-MS/MS data from *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare* controls were also analyzed to identify mycoplasma proteins. The resulting protein and peptide identification data are presented in Tables S5 and S6, respectively. Fig. 2B shows the overall numbers of mycoplasma proteins identified in the supernatants of infection cultures and shared or not with the corresponding control. For both *M. hyopneumoniae* strains, the number of proteins identified in the control samples (Tables S5A and C) were larger than that in infection cultures (Tables S5B and D). For *M. flocculare*, on the other hand, a larger number of proteins (81 proteins) was detected in the infection sample, in comparison to the corresponding control sample (67 proteins) (Tables S5E and F).

3.4. Secretion pathway predictions for swine and mycoplasma proteins identified in culture supernatants

In silico predictions were performed in order to provide clues on the secretion pathways of swine and mycoplasma proteins identified in supernatants from NPTr control, NPTr-Mh7448, NPTr-MhJ and NPTr-Mf cultures. Regarding swine proteins (Fig. 3A, Table S7), secretory pathway predictions were possible for about two thirds of the identified proteins, for the control and also for the infection cultures. About a third of the identified proteins (from 28.5% to 33.9%) was classified as ‘non-determined’, regarding secretion. Comparing the fractions of proteins with prediction of secretion pathways between the control and the infection cultures, it was noticeable a similar proportion (around 40% vs. 36.5%) of ‘extracellular vesicle’ prediction between them. The ‘classical’ fraction was similar among the control and the NPTr-Mh7448 and NPTr-MhJ cultures (~15–16%), but was slightly reduced in NPTr-Mf cultures (12%). Finally, the ‘non classical’ fraction was larger in the control NPTr culture in comparison to the infection cultures (18.4% vs. 5.7%–14.2%).

For mycoplasma proteins (Fig. 3B, Table S8), the ‘extracellular vesicles’ fraction was absent, and variable proportions of proteins assigned as of ‘classical’, ‘non classical”, and ‘non-determined” secretion were observed, either when comparing each infection sample with the corresponding control, or when comparing the three infection samples to each other. However, it was noticeable that, for both *M. hyopneumoniae* strains, the proportion of proteins predicted as secreted by the classical pathway was almost twice that of the corresponding controls.

3.5. Swine proteins secreted in response to M. hyopneumoniae 7448 infection

In order to understand the swine cell response to the pathogenic *M. hyopneumoniae* 7448, the 488 swine proteins identified in supernatants of NPTr-Mh7448 cultures were compared to the 287 proteins identified in the NPTr control culture (Fig. 2A). These two sets of proteins share 237 of them, with 251 and 50 proteins detected solely in the NPTr-Mh7448 sample and in the control, respectively. From the set of 237 shared proteins, 85 were differentially abundant, being 44 overrepresented in NPTr-Mh7448 supernatants and 41 overrepresented in the supernatant of the NPTr control (Table S9).

For functional analyzes, the sets of exclusively detected and overrepresented proteins in each sample were merged as a single set of differential proteins, with that of the NPTr control comprehending 91 proteins, and that of NPTr-Mh7448 supernatants comprehending 295 proteins. Functional inferences were established based on GO enrichment for the 85 differential proteins of the NPTr control (Table S10A) and for the differential 278 NPTr-Mh7448 proteins (Table S10B) into ‘biological process’ (BP), ‘cellular component’ (CC), and ‘molecular function’ (MF) categories. No GO annotations were retrieved for 6 NPTr control proteins and for 17 NPTr-Mh7448 proteins. Redundant GO subcategories were removed using REVIGO (Table S10C-10H) and plots of the resulting subcategory profiles of NPTr control and NPTr-Mh7448 samples are shown in Fig. S3. Several functional subcategories regarded as general were found as more enriched in both samples from BP, CC and MF categories. Some functional subcategories were exclusively found as overrepresented in NPTr control and NPTr-Mh7448 samples. For the NPTr control, exclusive subcategories were only general or redundant ones, for all three categories (BP, CC and MF).

For NPTr-Mh7448 proteins, the exclusive GO subcategories included not only general ones, but also subcategories that may be related to response to infection, as the 'cell death', ‘regulation of programmed cell death’, ‘NIK/NF-kappaB signaling’, and ‘antigen processing and presentation of exogenous peptide antigen’ BP subcategories, and the ‘proteasome complex’ CC subcategory. Table 1 shows the list of NPTr-Mh7448 proteins assigned to these subcategories, which includes damage-associated molecular patterns (DAMPs) proteins, namely high mobility group protein B2, heat shock protein HSP 90-alpha, and heat shock protein HSP 90-beta, assigned to the ‘cell death’ and/or ‘regulation of programmed cell death’ subcategories.

3.6. Differences between swine secreted proteins in response to M. hyopneumoniae 7448 and M. hyopneumoniae J infection

In order to understand the differences between the swine cell response to the pathogenic *M. hyopneumoniae* 7448 and that to the non-pathogenic strain *M. hyopneumoniae* J, the 488 swine proteins identified in supernatants of NPTr-Mh7448 cultures were compared to the 371 swine proteins identified in the supernatants of NPTr-MhJ cultures. These two sets of proteins shared 338 of them, with 151 and 34 being detected solely in the NPTr-Mh7448 and NPTr-MhJ samples, respectively. From the set of 338 shared proteins, 22 were differentially abundant, being 8 overrepresented in NPTr-Mh7448, and 11 overrepresented in the supernatant of the NPTr-MhJ (Table S11). Functional inferences were established based on the GO enrichment for the 159 NPTr-Mh7448 (Table S12A) and 45 NPTr-MhJ proteins (Table S12B) differential protein sets. For 11 NPTr-Mh7448 proteins and 8 NPTr-MhJ proteins no GO annotations were retrieved. Redundant GO subcategories were removed using REVIGO (Table S12C-12H) and plots of the resulting subcategory profiles of NPTr-Mh7448 and NPTr-MhJ samples are shown in Fig. S4. Several functional subcategories regarded as general were found as more enriched in both samples in the BP, CC and MF categories.

Some exclusive subcategories that may be related to response to infection with pathogenic *M. hyopneumoniae* 7448 were found for NPTr-

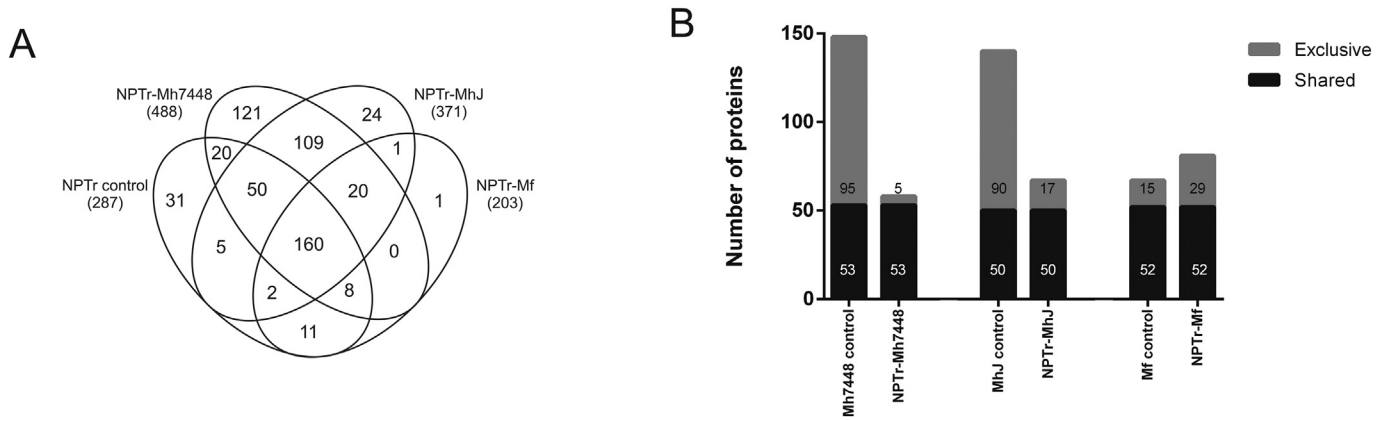

Fig. 2. Overview of the swine and mycoplasma proteins identified in NPTr-Mh7448, NPTr-MhJ, NPTr-Mf cultures, and NPTr and mycoplasma control cultures. (A) Venn diagram of swine proteins identified in NPTr control, NPTr-Mh7448, NPTr-MhJ, and NPTr-Mf cultures. Total numbers of proteins are indicated for each sample outside the diagram and the numbers of proteins exclusively detected in each sample or shared between them are indicated within the diagram. (B) Graphic of mycoplasma proteins identified in *M. hyopneumoniae* 7448 (Mh7448) control, NPTr-Mh7448, *M. hyopneumoniae* J (MhJ) control, NPTr-MhJ, *M. flocculare* (Mf) control and NPTr-Mf cultures. Exclusive proteins are in grey color and number of exclusive proteins are shown inside of bar or above. Shared proteins between control and after NPTr cell infection from the same mycoplasma specie/strain are shown in black and number of proteins are shown inside of bar.

7448 proteins. These subcategories are related to cell death and comprehend 'cell death', 'regulation of cysteine-type endopeptidase activity involved in execution phase of apoptosis' and 'proteasome complex'. Table 2 shows the list of NPTr-Mh7448 proteins assigned to these subcategories. In contrast, for NPTr-MhJ proteins, only general subcategories were found as exclusive (see Table S12B).

3.7. Differences between swine proteins secreted in response to M. hyopneumoniae 7448 and M. flocculare infection

In order to understand the differences in swine cell response to the pathogenic *M. hyopneumoniae* 7448 and to the commensal *M. flocculare*, the 488 swine proteins identified in supernatants of NPTr-Mh7448 cultures were compared to the 203 identified in the NPTr-Mf supernatants. These two sets of proteins share 187 of them, with 301 and 16 being detected solely in NPTr-Mh7448 and NPTr-Mf samples, respectively. From the set of 187 shared proteins, 53 were differentially abundant, being 27 overrepresented in NPTr-Mh7448 supernatants and 26 overrepresented in NPTr-Mf supernatants (Table S13). Functional inferences were established based on the GO enrichment for the 328 NPTr-Mh7448 (Table S14A) and 42 NPTr-Mf (Table S14B) differential protein sets. No GO annotations were retrieved for 23 NPTr-Mh7448 proteins and for two NPTr-Mf proteins. Redundant GO subcategories were removed using REVIGO (Table S14C-14H) and plots of the resulting subcategory profiles of NPTr-Mh7448 and NPTr-Mf samples are shown in Fig. S5.

Several functional subcategories regarded as general were found as more enriched in both samples in BP, CC, and MF categories. The functional enrichment profile was almost identical to that obtained from the comparison between swine proteins from NPTr-Mh7448 supernatants and the NPTr control supernatant. Indeed, the BP subcategories 'NIK/NF-kappaB signaling', 'antigen processing and presentation of exogenous peptide antigen', 'cell death' and 'regulation of programmed cell death' were also found as exclusive for NPTr-Mh7448 when compared with NPTr-Mf. Moreover, the proteins assigned to the 'NIK/NF-kappaB signaling', 'antigen processing and presentation of exogenous peptide antigen', 'cell death', 'regulation of programmed cell death' and 'proteasome complex' subcategories were the very similar to that listed in Table 1. Regarding the MF category, the few subcategories that were exclusive for NPTr-Mh7448 proteins in comparison to NPTr-Mf proteins were only general and redundant ones. For the NPTr-Mf proteins, as observed for the NPTr control, exclusive subcategories were also general or redundant ones for all three categories (BP, CC and MF).

3.8. Identification and comparison of protein co-expression clusters from NPTr-Mh7448, NPTr-MhJ or NPTr-Mf supernatants

To explore the interactions among the swine identified proteins, co-expression networks were generated for the protein sets from NPTr-Mf, NPTr-MhJ, and NPTr-Mh7448 supernatants (Fig. S6). The NPTr-

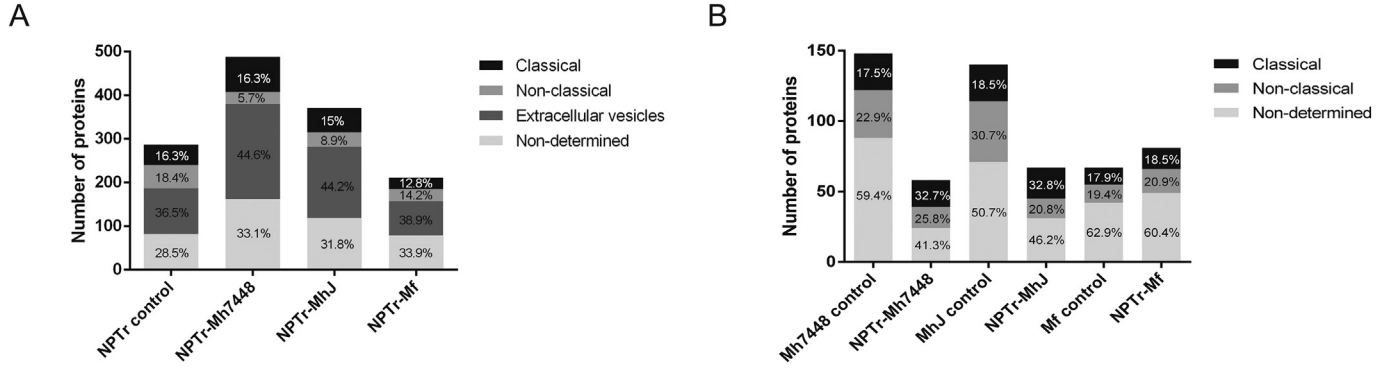

Fig. 3. Predicted secretory pathways of swine and mycoplasma proteins identified in NPTr-Mh7448, NPTr-MhJ, NPTr-Mf cultures, and NPTr and mycoplasma control cultures. Percentages of proteins with prediction of secretion by classical, non-classical or extracellular vesicles. Remaining proteins were classified as non-determined. (A) Swine proteins identified in NPTr control, NPTr-Mh7448, NPTr-MhJ, and NPTr-Mf cultures. (B) Mycoplasma proteins identified in *M. hyopneumoniae* 7448 (Mh7448) control, NPTr-Mh7448, *M. hyopneumoniae* J (MhJ) control, NPTr-MhJ, *M. flocculare* (Mf) control and NPTr-Mf cultures.

Table 1
Proteins assigned to functional subcategories exclusively overrepresented in NPTr-Mh7448 in comparison to the NPTr control.

Functional annotation (Subcategories)[a]		Protein name	Acession number[b]	Fold change	*p* value
BP	NIK/NF-kappaB signaling & Antigen processing and presentation of exogenous peptide antigen	26S protease regulatory subunit 8 isoform X2	XP_013836293.1	–	–
		Proteasome activator 28 alpha subunit	AAQ83574.1	–	–
		Proteasome subunit beta type-5	XP_001924480.2	–	–
		26S protease regulatory subunit 7	NP_001231937.1	–	–
		26S proteasome non-ATPase regulatory subunit 11	XP_013845409.1	–	–
		Proteasome activator complex subunit 2	NP_999444.1	–	–
		Proteasome subunit alpha type-7	NP_001230581.1	–	–
	Regulation of programmed cell death	Acidic leucine-rich nuclear phosphoprotein 32 family member B	XP_020922136.1	–	–
		T-complex protein 1 subunit alpha	NP_001230356.1	–	–
		Heat shock protein HSP 90-beta isoform X1	XP_005666120.1	1.7	0.045
		Proliferation-associated protein 2G4	XP_013843758.1	–	–
		Proteasome activator complex subunit 3	NP_999513.1	–	–
		40S ribosomal protein S7	XP_005662840.1	–	–
		Lactoylglutathione lyase	XP_001927992.1	–	–
		26S protease regulatory subunit 8 isoform X2	XP_013836293.1	–	–
		Dynamin-1-like protein	XP_013853201.1	–	–
		Valosin precursor	1303334A	2.1	0.019
		Serine/threonine-protein kinase PAK 2	XP_020925795.1	–	–
		High mobility group protein B2	NP_999228.1	–	–
		m7GpppX diphosphatase	NP_998955.1	–	–
		Cystathionine gamma-lyase	NP_001038050.1	–	–
		Protein SET	NP_001231019.1	–	–
		Notchless protein homolog 1 isoform X1	XP_003358201.1	–	–
		Asparagine synthetase	NP_001161112.1	–	–
		Catenin alpha-1 isoform X1	XP_003124036.3	–	–
		Serine/threonine-protein phosphatase 2A 65 kDa Regulatory subunit A alpha isoform	NP_999189.1	–	–
		Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	NP_001038024.1	–	–
		Glutamate–cysteine ligase regulatory subunit	XP_001926413.2	–	–
		Superoxide dismutase 1 [Cu-Zn]	AHW83665.1	–	–
		40S ribosomal protein S3	XP_013834591.1	–	–
		LOW QUALITY PROTEIN: DNA damage-binding protein 1	XP_003122699.2	–	–
		Peptidyl-prolyl cis-trans isomerase D	NP_001231315.1	–	–
		Heme oxygenase 1	NP_001004027.1	–	–
		Nucleoside diphosphate kinase A isoform X1	XP_005668988.1	–	–
	Cell death	Heat shock protein HSP 90-alpha	NP_999138.1	2.7	0.011
		Dehydrogenase, glyceraldehydephosphate	681085A	–	–
		Glyceraldehyde-3-phosphate dehydrogenase	NP_001193288.1	3.0	0.001
		Acidic leucine-rich nuclear phosphoprotein 32 family member B	XP_020922136.1	–	–
		Proteasome activator complex subunit 3	NP_999513.1	–	–
		Dynamin-1-like protein	XP_013853201.1	–	–
		Valosin precursor	1303334A	2.1	0.019
		Serine/threonine-protein kinase PAK 2	XP_020925795.1	–	–
		High mobility group protein B2	NP_999228.1	–	–
		Notchless protein homolog 1 isoform X1	XP_003358201.1	–	–
		Glutamate–cysteine ligase regulatory subunit	XP_001926413.2	–	–
		Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	NP_999189.1	–	–
		40S ribosomal protein S3	XP_013834591.1	–	–
		Peptidyl-prolyl cis-trans isomerase D	NP_001231315.1	–	–
		Heme oxygenase 1	NP_001004027.1	–	–
		DNA replication licensing factor MCM2	XP_003483287.1	–	–

(*continued on next page*)

Table 1 (*continued*)

Functional annotation (Subcategories)[a]		Protein name	Acession number[b]	Fold change	*p* value
CC	Proteasome complex	Proteasome subunit beta type-4	NP_001231384.1	–	–
		Proteasome subunit beta type-3	NP_001138374.1	–	–
		26S proteasome non-ATPase regulatory subunit 4	NP_001038000.1	–	–
		Proteasome subunit alpha type-3	XP_001928025.1	–	–
		Proteasome (prosome, macropain) subunit, beta type, 2	JAG69251.1	–	–
		Proteasome activator 28 alpha subunit	AAQ83574.1	–	–
		26S proteasome non-ATPase regulatory subunit 2	NP_001230406.1	–	–
		26S protease regulatory subunit 7	NP_001231937.1	–	–
		Proteasome subunit alpha type-4	NP_001231397.1	–	–
		26S proteasome non-ATPase regulatory subunit 11	XP_013845409.1	–	–
		Proteasome activator complex subunit 3	NP_999513.1	–	–
		Proteasome subunit alpha type-7	NP_001230581.1	–	–
		26S proteasome non-ATPase regulatory subunit 5	XP_020923822.1	–	–
		Proteasome subunit beta type-1	NP_001231282.1	–	–
		26S protease regulatory subunit 8 isoform X2	XP_013836293.1	–	–
		Proteasome subunit beta type-5	XP_001924480.2	–	–
		Proteasome subunit beta type-6	NP_001138398.2	–	–
		Proteasome subunit alpha type-1	XP_003123013.1	–	–
		Proteasome subunit alpha type-6	NP_001132944.1	–	–
		Proteasome activator complex subunit 2	NP_999444.1	–	–
		Valosin precursor	1303334A	2.1	0.019

[a] Functional annotation subcategories of Biological Process (BP) or Cellular Component (CC) categories according to Gene Ontology.
[b] Acession number retrieved from NCBI (https://www.ncbi.nlm.nih.gov).

Table 2
Proteins assigned to functional subcategories exclusively overrepresented in NPTr-Mh7448 in comparison to the NPTr-MhJ.

Functional annotation (Subcategories) [a]		Protein name	Acession number [b]	Fold change	*p* value
BP	Regulation of cysteine-type endopeptidase activity involved in execution phase of apoptosis	40S ribosomal protein S3	XP_013834591.1	–	–
		serine/threonine-protein kinase PAK 2	XP_020925795.1	–	–
	Cell death	DNA replication licensing factor MCM2	XP_003483287.1	–	–
		dynamin-1-like protein	XP_013853201.1	–	–
		acidic leucine-rich nuclear phosphoprotein 32 family member B	XP_020922136.1	–	–
		heme oxygenase 1	NP_001004027.1	–	–
		serine/threonine-protein kinase PAK 2	XP_020925795.1	–	–
		dehydrogenase,glyceraldehydephosphate	681085A	–	–
		notchless protein homolog 1 isoform X1	XP_003358201.1	–	–
		40S ribosomal protein S3	XP_013834591.1	–	–
		proteasome activator complex subunit 3	NP_999513.1	–	–
CC	Proteasome complex	26S proteasome non-ATPase regulatory subunit 4	NP_001038000.1	–	–
		proteasome subunit alpha type-3	XP_001928025.1	–	–
		proteasome subunit beta type-5	XP_001924480.2	–	–
		26S proteasome non-ATPase regulatory subunit 11	XP_013845409.1	–	–
		proteasome activator complex subunit 3	NP_999513.1	–	–

[a] Functional annotation subcategories of Biological Process (BP) or Cellular Component (CC) categories according to Gene Ontology.
[b] Acession number retrieved from NCBI (https://www.ncbi.nlm.nih.gov)

Mh7448 network has 264 nodes, 1579 connections and 15 clusters (Fig. S6A); the NPTr-MhJ network has 270 nodes, 2554 connections and 8 clusters (Fig. S6B); and the NPTr-Mf network has 273 nodes, 4565 connections and 10 clusters (Fig. S6C). Therefore, although the number of nodes (proteins) present in the three networks were similar, the differences in the protein expression correlation values led to variation in the number of identified clusters in each of them.

Functional enrichment of clustered protein sets was performed to infer the main function of each cluster (Fig. S6; Tables S15). Overall, 21 functions were enriched, with only part of them shared between the analyzed networks. Three functions were common to all three networks, namely 'translation', 'cell development' and 'response to stimulus'. Regarding exclusive clusters, 8 were found only for the NPTr-Mh7448 network, while only one and three, respectively, were found for the NPTr-MhJ and NPTr-Mf networks. Interestingly, 'bacterial invasion of epithelial cells' infection-related cluster was found as exclusive for the NPTr-Mh7448 network.

3.9. Potential mycoplasma virulence factors differentially represented in NPTr cell cultures infected with M. hyopneumoniae 7448, M. hyopneumoniae J or M. flocculare

In order to identify potential mycoplasma virulence factors being secreted in response to the contact with NPTr cells, mycoplasma proteins identified in NPTr-Mh7448, NPTr-MhJ, and NPTr-Mf supernatants were compared to those identified in the supernatants of *M. hyopneumoniae* 7448, *M. hyopneumoniae* J *or M. flocculare* control cultures, respectively. The differentially represented mycoplasma proteins found in the three pairwise comparisons are listed in Table S16. *M. hyopneumoniae* 7448 proteins were identified as potential virulence factors according to Ferreira & de Castro [51], and their *M. hyopneumoniae* J and *M. flocculare* orthologs were also assumed as such. Table 3 shows the list of potential virulence factors detected for each mycoplasma in the infection assay samples (NPTr-Mh7448, NPTr-MhJ and NPTr-Mf).

LC-MS/MS analyses of *M. hyopneumoniae* 7448 revealed 23 (out of 58) proteins differentially represented in NPTr-Mh7448 in comparison to control. Among these proteins, 5 were exclusively detected in NPTr-Mh7448, 18 overrepresented in the NPTr-Mh7448 supernatant (Table

Table 3
Virulence factor proteins identified by LC-MS/MS in NPTr-Mh7448, NPTr-MhJ or NPTr-Mf supernatants.

Protein name [a]	Acession number [b]		
	M. hyopneumoniae 7448	*M. hyopneumoniae* J	*M. flocculare*
Adhesins			
Adhesin like-protein P146	AAZ54025.1	AAZ44746.1	WP_002557493.1
Lppt protein	AAZ53743.1	AAZ44456.1	WP_002557448.1
Protein P102 - copy 1	AAZ53573.1	AAZ44286.1	ND
Protein P102 - copy 2	AAZ53482.1	ND	ND
Protein P97 - copy 1	AAZ53572.1	ND	ND
Protein P97 - copy 2	AAZ53483.1	AAZ44197.1	WP_002557588.1
p76 membrane protein precursor	AAZ53863.1	AAZ44580.1	ND
Prolipoprotein p65	AAZ54018.1	AAZ44739.1	ND
Outer membrane protein - P95	AAZ53476.1	ND	WP_002557580.1
Putative p216 surface protein	AAZ53862.1	AAQ11195.1	WP_002558000.1
L-lactate dehydrogenase	AAZ53511.1	ND	ND
Lipoproteins			
Lipoprotein	AAZ53591.2	AAZ44304.2	ND
Lipoprotein	AAZ53871.1	ND	ND
Membrane nuclease, lipoprotein	ND	AAZ44665.1	ND
Putative lipoprotein	ND	AAZ44462.1	ND
Oxidative stress-related proteins			
Putative thioredoxin	ND	AAZ44468.1	ND
2-Cys peroxiredoxin	ND	AAZ44187.2	WP_002557577.1
Hypothetical proteins			
Hypothetical protein	WP_044272653.1	ND	ND
Hypothetical protein	WP_044272715.1	ND	ND
Hypothetical protein	WP_044272522.1	ND	ND
Hypothetical protein MHP7448_0662	AAZ54024.2	AAZ44745.2	ND
Antigenic proteins			
46 kDa surface antigen	ND	P0C0J8.1	ND
Ribonucleoside-diphosphate reductase beta chain	ND	ND	WP_002557765.1
Others			
ABC transporter xylose-binding lipoprotein	ND	AAZ44690.2	ND
Acetate kinase	ND	AAZ44591.1	WP_002557640.1
ATP synthase alpha chain	ND	AAZ44562.1	ND
Nucleotide exchange factor GrpE	ND	ND	WP_002557468.1
PTS IIA component	ND	ND	WP_002557450.1
Trigger factor	ND	AAZ44236.2	WP_002557794.1

[a] Protein name according *M. hyopneumoniae* 7448 acession number.
[b] Acession number retrieve from NCBI (https://www.ncbi.nlm.nih.gov); ND - not detected.

S16A). Among the differentially represented proteins in NPTr-Mh7448 supernatant (5 exclusive and 18 overrepresented), 17 are known virulence factors (Table 3) and 4 have unknown functions (hypothetical proteins). For *M. hyopneumoniae* J, 35 proteins were differentially represented in NPTr-MhJ in comparison to control, being 17 exclusive and 18 overrepresented proteins (Table S16B). In the set of differentially represented proteins in NPTr-MhJ supernatant, 21 were virulence factors, from which 10 were also overrepresented in NPTr-Mh7448 supernatant (Table 3). Finally, 36 *M. flocculare* proteins were differentially represented in NPTr-Mf, being 29 exclusive and 7 overrepresented in comparison to control (Table S16C). In the set of differentially represented proteins in NPTr-Mf supernatants, 12 proteins were virulence factors, from which 5 were also overrepresented in NPTr-Mh7448 supernatant (Table 3).

4. Discussion

Secreted proteins can reflect a broad variety of different conditions of the cell, and cell secretion responds to both intracellular factors and external stimuli. The cell secretome mediates a plethora of extracellular functions, depending on the cell itself and its environment. In host-pathogen interactions, secreted proteins are the first line of interplay between the interacting organisms. Regarding the pathogen, secreted proteins may be important virulence factors or pathogenicity determinants. In the case of *M. hyopneumoniae*, for instance, secreted bacterial proteins may mediate immunoevasion, contributing to bacterial survival, or trigger pro-inflammatory mechanisms, contributing to the PEP severity [21,52]. From the host point of view, considering swine infections by *M. hyopneumoniae*, host secreted proteins may mediate both a protective immune response, leading to pathogen elimination, or an exacerbated inflammatory response, that causes PEP lung lesions [53]. Therefore, the outcome of a swine *M. hyopneumoniae* infection depends on a delicate balance between pathogenesis determinants from both bacterial and host origins, with secreted proteins being important players.

Identification of secreted proteins in cell cultures is challenging due to the presence of serum proteins from fetal bovine serum added to the cell culture media. Overrepresented serum proteins are known to impose difficulties to the identification of less abundant proteins in culture supernatants by mass spectrometry [54]. Mammal cell culture in serum-deprived medium is a common strategy in most secretome studies to avoid this problem, although it may cause a decrease in cell proliferation [55,56]. In this work, NPTr cell proliferation was also affected by serum starvation, but cells remained viable, as shown by MTT assays. The NPTr cell line was chosen because it is from swine tracheal epithelium [30], and, therefore, their cultivation along with *M. hyopneumoniae* and *M. flocculare* would be representative of host-bacteria interactions in natural mycoplasma respiratory infections.

There is no consensual protocol for mammal cell culture in serum-free medium for secretome studies [57–62]. Therefore, prior to use in the infection assays and secretome surveys, different NPTr cell culture protocols were tested. In our standardized conditions, NPTr cell

proliferation was at its peak after 18 h of culture, and, therefore, for the infection assays, swine cells were incubated with the mycoplasmas for this period of time. The changes in morphology observed in NPTr cells while infected with mycoplasmas were indicative of differential responses, as these changes were more evident in the NPTr cells infected with the pathogenic *M. hyopneumoniae* 7448.

Protein samples recovered from culture supernatants showed high reproducibility among biological replicates in this work, which was initially evidenced by their similar electrophoretic profiles, and later confirmed by the LC-MS/MS results. The percentage of common proteins identified in technical replicates ranged from 75% to 95% (for both swine and mycoplasma proteins) and > 90% of the proteins were identified in the three biological replicates of each of the analyzed supernatant samples. Corresponding bands in the SDS-PAGE protein profiles of NPTr control, NPTr-Mh7448 and NPTr-MhJ supernatants were likely the result of residual FBS proteins contamination and/or cell death due to serum deprivation, as previously noticed [63]. The performed immunoblots to assess the relative abundance of the intracellular markers β-actin and calnexin in cellular and supernatant fractions of NPTr control, NPTr-Mh7448, NPTr-MhJ and NPTr-Mf cultures further demonstrated that only relatively minor amounts of intracellular proteins were in the extracellular fraction. The detection of some β-actin in supernatants was expected, as this is a quite abundant intracellular protein. Calnexin, on the other hand, which was used as low abundant intracellular marker, was not detected in any of the supernatants. Despite all precautions, the contamination of secretome samples by intracellular and serum proteins cannot be completely prevented [54,64].

Here, a comprehensive survey of the NPTr cell secretome was performed, with the identification and relative quantification of 598 swine proteins, considering both control and infection assay samples. A fraction of ~26% of these proteins (160 proteins) was shared by all supernatant samples, and this set of common proteins included the more abundant ones, like some proteins involved with binding (retinol-binding protein, galectin, and 14–3-3), biological regulators (thymosin, peptidyl-prolyl cis-trans isomerase A, alpha-enolase, S100-A11 and annexin A8) and cytoskeletal proteins (actin, vimentin, and profilin). As most of these proteins are typically intracellular, their presence in the supernatants may be, at least in part, the result of cell death, as discussed above. However, for at least 10 of these proteins, there are evidences of 'non-classical' or 'extracellular vesicle' secretion. A fraction of ~5% of the overall number of swine proteins detected in our assays (35 out of 598 proteins) were assumed to be FBS contaminants. This assumption was based in the fact that, all these proteins were detected in at least one of the control mycoplasma cultures. These protein set included well-known serum proteins such as albumin, alpha-glycoprotein, apolipoprotein, microglobulins, hemoglobins and others [65], which were excluded from all further secretome analyses.

Proteins can be released to the extracellular space through various mechanisms, including classical secretion, for proteins with signal peptides, and non-classical secretion, mediated by recycling endosomes or extracellular vesicles. Most swine proteins identified in this study were predicted as secreted by one of these pathways. Overall NPTr secretome pathway profiles were altered in all infection assays in comparison to that of the NPTr control. Interestingly, secretion by extracellular vesicles was more common (> 44%) among swine proteins secreted in response to *M. hyopneumoniae* (for both 7448 and J strains), in comparison to swine proteins secreted in response to *M. flocculare* or to NPTr control samples. This is consistent with previous results showing the presence of a mycoplasma can modify vesicle production by mammal host cells [66,67].

A larger set of swine secreted proteins (488) was observed in response to *M. hyopneumoniae* 7448, in comparison to those observed in response to *M. hyopneumoniae* J (371 proteins) and *M. flocculare* (203 proteins). Overall, the set of 203 swine proteins identified in NPTr-Mf supernatants was ~89% shared with that of the NPTr control, denoting little alteration in the NPTr secretion profile in response to this non-pathogenic species. On the other hand, from the set of 488 swine proteins identified in NPTr-Mh7448 supernatants, only 238 proteins (~48%) were shared with the NPTr control, which shows, in contrast to NPTr-Mf supernatants, an overwhelming change in the NPTr cell secretion profile in response to the pathogenic *M. hyopneumoniae* 7448. The non-pathogenic *M. hyopneumoniae* J, in turn, induced an intermediary response in terms of secreted proteins (371 proteins, with ~58% of them shared with the NPTr control).

In order to gain insights in swine cell behavior upon *M. hyopneumoniae* infection, swine proteins identified in NPTr-Mh7448 supernatants were compared to those identified in NPTr control, NPTr-MhJ or NPTr-Mf supernatants. Distinct profiles of overrepresented and underrepresented functional subcategories were obtained based on proteins with differential abundances between samples. The few (only 5) and general underrepresented categories (data not shown) were not considered and were not further analyzed. On the other hand, among the hundreds of overrepresented ones, five of those exclusively enriched for NPTr-Mh7448 proteins were related to the swine cell response to the pathogen, namely 'cell death', 'regulation of programmed cell death', 'NIK/NF-kappaB signaling', 'antigen processing and presentation of exogenous peptide antigen' and 'proteasome complex'. In contrast, those exclusively enriched for NPTr control, NPTr-MhJ and NPTr-Mf proteins were mostly related to general cell functions. Such differential and specific swine cell response to *M. hyopneumoniae* 7448 involving secreted proteins is being described for the first time. The absence of functional subcategories involved with pathogenicity after NPTr cells infection with *M. hyopneumoniae* J or *M. flocculare* corroborates that the model of infection assay proposed in this study reproduces, at least in part, the natural situation in the swine respiratory epithelium.

The enrichment of 'cell death' and 'regulation of programmed cell death' subcategories in response to *M. hyopneumoniae* 7448 is in agreement with the cell death events that naturally occur in infected lung tissues during PEP [68]. Most of the proteins included in these subcategories had a secretory pathway predicted as 'non-classical' or 'extracellular vesicle' and some are well known DAMPs [69], as high mobility group protein B2, heat shock protein HSP 90-beta, and heat shock protein HSP 90-alpha. Once exposed or released in the extracellular milieu, DAMPs are capable of acting as "danger signals" and stimulate pro-inflammatory response upon interaction with a wide range of receptors on host immune cells [69,70]. Via DAMPs, the immune system can respond not only to an infection per se, but also to non-physiological cell death, damage or stress [71]. Secretion of DAMPs by *M. hyopneumoniae*-induced necrotic cells could explain the increase of pro-inflammatory cytokines observed in PEP lung lesions [25,28,72].

The other subcategories exclusively enriched for NPTr-Mh7448 proteins ('NIK/NF-kappaB signaling', 'antigen processing and presentation of exogenous peptide antigen', and 'proteasome complex') were enriched due to the detection and abundance of proteasome proteins. Host NF-kappaB pathway activation by *M. hyopneumoniae* has been already demonstrated [72]. Moreover, proteasome proteins have been reported as enriched in endothelial microparticles (EMPs) secreted by agonist-stimulated endothelial cells [73]. Formation and secretion of EMPs have been associated with cell dysfunction, stress and injury, and can be induced by several factors, including bacterial lypopolysaccharides and toxins [74]. Extracellular proteasome protein components have also been identified in the extracellular space in healthy individuals, but their increase has been correlated with disease progression in patients suffering different diseases [75,76]. A similar scenario was evidenced by our results, as proteasome proteins were identified in all NPTr control and infection samples, but their number and abundance were increased in *M. hyopneumoniae* 7448 cultures. The role of extracellular proteasome protein in the context of PEP remain to be investigated.

The WGCNA co-expression methodology is typically used for

exploring correlation between gene or protein expression levels [47]. The built co-expression networks provided further evidences of functional differences between the profiles of NPTr proteins secreted in response to *M. hyopneumoniae* 7448, *M. hyopneumoniae J* and *M. flocculare*. The 'bacterial invasion of epithelial cells' cluster, found as exclusive for the NPTr-Mh7448 set of secreted proteins reinforces the idea of a NPTr cell response differential and specific to these pathogenic strain (in comparison to that to *M. hyopneumoniae* J, or to *M. flocculare*). This GO category comprehends not only proteins actually involved in host cell invasion, but also proteins involved in other bacterial infection mechanisms. Regarding the host cell invasion issue, *M. hyopneumoniae* and *M. flocculare* are usually described as extracellular species, but some recent studies have provided evidences that *M. hyopneumoniae*, upon interaction with surface accessible actin on the epithelial cells, may be phagocytosed and survive within phagolysosomes [77–79].

Along with secreted swine factors, secreted bacterial proteins in response to interaction to host cells likely contribute to the differences in pathogenicity between *M. hyopneumoniae* strains or between *M. hyopneumoniae* and *M. flocculare*. Differences between the *M. hyopneumoniae* and *M. flocculare* secretomes have been found in a previous proteomic survey of broth culture supernatants [21]. Here, overall totals of 153, 157, and 96 proteins were identified for *M. hyopneumoniae 7448*, *M. hyopneumoniae J*, and *M. flocculare*, respectively, considering both infection assays and control cultures. As expected, these numbers were higher than those obtained for cultures in the presence of FBS [21], even in the presence of swine proteins secreted by NPTr cells. A larger number of proteins was identified in the mycoplasma control cultures. This may be due to the lack of quenching by swine proteins, which occur in NPTr cell infections. Another not mutually exclusive explanation would be a certain degree of mycoplasma cell death in cultures in the absence of any swine factor [80], which is corroborated by the detection of bacterial ribosomal proteins in control mycoplasma cultures but not in the NPTr cell infections.

Proteins previously assigned as virulence factors of *M. hyopneumoniae* 7448 [51] and their orthologs in *M. hyopneumoniae* J or *M. flocculare* were identified as being secreted during NPTr infection. *M. hyopneumoniae* 7448 secreted a higher number of adhesins (11) than *M. hyopneumoniae* J (7) and *M. flocculare* (5). *M. hyopneumoniae* 7448 also secreted more hypothetical proteins described as virulence factors during NPTr cell infection, suggesting that these unknown proteins are important for *M. hyopneumoniae* pathogenesis. On the other hand, oxidative-stress related proteins, some enzymes (e.g. acetate kinase and ATP synthase), and some antigenic proteins (e.g. P46) were identified as being secreted by *M. hyopneumoniae J* or *M. flocculare*, but not by *M. hyopneumoniae* 7448. In this case, although immunogenic [51], they may not contribute significantly to pathogenicity. The identified mycoplasma secreted proteins also had their secretory pathway predicted. The lack of a bacterial extracellular vesicle database, resulted in the assignment of several proteins to the 'non-determined' category, which was the largest for NPTr-Mf and all control samples. For NPTr-Mh7448 and NPTr-MhJ supernatants, most *M. hyopneumoniae* proteins could be assigned to the 'classical' or to the 'non-classical' pathways of secretion, with similar proportions between them.

5. Conclusion

In conclusion, this work presented, as far as we are aware of, for the first time, a comprehensive and reciprocal survey of secreted proteins of both swine and bacterial origin, using cultures of NPTr swine epithelial cell line infected with the pathogenic *M. hyopneumoniae* 7448 and a LC-MS/MS proteomic approach. Moreover, parallel comparative surveys were carried out with the same swine cells infected with the *M. hyopneumoniae* J strain, and the closely related *M. flocculare*, both non-pathogenic. Differences in the NPTr secretome profiles were observed in response to infection with each *M. hyopneumoniae* strain or *M. flocculare*. DAMPs and extracellular proteasome proteins, commonly secreted in response to cell injury and death, were identified as being secreted by NPTr cells only in response to *M. hyopneumoniae* 7448. Mycoplasma proteins secreted during NPTr cell infection were also different for each mycoplasma strain or species. All three mycoplasmas secreted known virulence factors during NPTr cell infection, but *M. hyopneumoniae* 7448 secreted a higher number of adhesins and many hypothetical proteins possibly related to its pathogenicity. Overall, the performed secretomic survey provided comprehensive lists of swine and mycoplasma proteins likely involved in host-bacterial interactions relevant for PEP. Some of these secreted proteins are expected to be interesting targets for additional studies aiming the development of novel methods for PEP prevention and treatment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.08.018.

Conflict of interest

The authors declare no conflict of interest.

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4.2 Análise proteômica das proteínas intracelulares de uma linhagem celular de traqueia suína infectada com a uma cepa patogênica de *M. hyopneumoniae.*

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Intracellular changes of swine tracheal cell line infected with a Mycoplasma hyopneumoniae *pathogenic strain*

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F.M.A.L.Z: delineamento experimental, execução de todos os experimentos e redação do manuscrito. H.M e J.R.B: auxílio nos experimentos de proteômica *bottom up* e revisão do manuscrito; H.B.F: delineamento experimental, análise e discussão dos resultados e revisão do manuscrito.

Intracellular changes of swine tracheal cell line infected with a *Mycoplasma hyopneumoniae* pathogenic strain

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Running title: Swine cell response to *Mycoplasma hyopneumoniae*

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Abstract

Mycoplasma hyopneumoniae is the etiological agent of enzootic pneumonia (EP), a widespread disease considered a major cause of economic loss in the pig industry. Due to the important role of swine host response in *M. hyopneumoniae* infection outcome, the whole proteome of NPTr swine epithelial cells infected with the *M. hyopneumoniae* pathogenic strain 7448 were analyzed by a LC-MS/MS approach to provide clues on intracellular changes in response to the pathogen. Overall, 853 swine protein species were identified, 156 of which were differentially represented in response to *M. hyopneumoniae* 7448 infection. These differentially represented proteins were functionally categorized, and 57 of them were assigned to immune system and/or response to stimulus functional subcategories. A comparative expression analysis of these immune-related proteins in NPTr cells infected with non-pathogenic mycoplasmas, as the *M. hyopneumoniae* J strain or *M. flocculare*, revealed proteins whose abundance is altered only in response to the pathogenic *M. hyopneumoniae* 7448. Among these proteins, there are calcium homeostasis and ER stress-related ones, providing evidence of molecular mechanisms altered in swine cells during EP.

INTRODUCTION

Mycoplasma hyopneumoniae is the etiological agent of enzootic pneumonia (EP), a highly prevalent disease that is a major cause of economic loss in the pig industry (Maes et al., 2017). EP causes reduced growth rate along with lower feed conversion efficiency in swine. *M. hyopneumoniae* can also potentiate disease caused by other porcine pathogens, either bacterial or viral ones, being one of the primary agents involved in the porcine respiratory disease complex (Thacker and Minion, 2010).

M. hyopneumoniae is an extracellular bacterium, that adheres to the host epithelium upon interaction with swine molecules as extracellular actin, glycosaminoglycans, heparin, fibronectin and plasminogen (Jenkins et al., 2006; Deutscher et al., 2010; Raymond et al., 2013; Raymond et al., 2014; 2015; Raymond et al., 2018). Adherence of *M. hyopneumoniae* to swine trachea ciliary cells results in ciliostasis, clumping, loss of cilia, and epithelial cell death (Debey et al., 1992). *M. hyopneumoniae* cytotoxicity has been associated to different factors, from blocking of Ca^{2+} channels (Park et al., 2002) to apoptosis induced by specific surface proteins, like lipid-associate membrane proteins (LAMPs) (Bai et al., 2015), or signal peptidase I (Paes, et al., 2017).

Swine host response is an important player in *M. hyopneumoniae* pathogenesis. Increase of pro-inflammatory cytokines induced by *M. hyopneumoniae* infection have been associated to swine lung lesions (Ahn et al., 2009). Infection with *M. hyopneumoniae* is often chronic, indicating bacteria immune evasion. It was demonstrated that *M. hyopneumoniae* suppresses the host immune systems by inhibiting macrophage-mediated phagocytosis and decreasing antibody production by B lymphocytes (Maes et al., 1996). These effects are observed in the early stages of the infection, although they may remain for several weeks after the initial infection. In this way, 'Omics' studies are expected to provide more clues on the complex host-pathogens interactions that occur during EP. For instance, more than 2000 genes had their transcription altered in pulmonary alveolar macrophages upon *M. hyopneumoniae* infection (Bin et al., 2014). Complementary proteomics studies can provide additional and valuable information, considering that some *M. hyopneumoniae*-induced alterations in swine cells may occur or be evident only at the protein level.

In the present study, the total cell lysate of NPTr cells infected with *M. hyopneumoniae* 7448 were analyzed, in order to identify swine proteins differentially

represented in response to *M. hyopneumoniae* infection. A swine epithelial cell line is being used to provide clues on initial alterations in response to local infection. A liquid chromatography (LC)-tandem mass spectrometry (MS/MS) proteomic approach was used, and more than 150 swine proteins presented differential abundance in infected NPTr cells in comparison to the non-infected control, many of them related to immune response. We also demonstrated that these immune-related proteins have their abundance altered in response to *M. hyopneumoniae* 7448, but not to the non-pathogenic *M. hyopneumoniae* J strain or the commensal *Mycoplasma flocculare*.

METHODS AND MATERIALS

Mycoplasma cultures

M. hyopneumoniae 7448 was originally isolated from a naturally infected pig from Lindóia do Sul (SC, Brazil) and its pathogenicity was experimentally demonstrated (Vasconcelos et al., 2005) . *M. hyopneumoniae* 7448, *M. hyopneumoniae* J or M. *flocculare* were cultured in Friis medium (Friis, 1971) containing 25% of fetal bovine serum (FBS; Gibco™, Thermo Fisher Scientific, IL, USA), in 25 ml batches, at 37ºC for 48 h with agitation, as previously described (Leal et al., 2016). After culture, mycoplasma cells were centrifuged at 3500 g for 15 min, washed three times with PBS pH 7.4, for medium removal, and resuspended in 5 ml of Minimal Essential Medium with Earle's Balanced Salt Solution and L-glutamine (MEM/EBSS; LGC Biotecnologia, SP, Brazil), prior to use in infection assays.

Porcine cell cultures

Newborn Porcine Trachea (NPTr) cells (Ferrari et al., 2003) were purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy), and cultured in MEM/EBSS supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO^2. Approximately 5 x 10^5 cells were seeded in 75 cm^3 plates and grown for 48 h until 60-70% confluence. After 48 h, cells were washed five times with serum free-MEM/EBSS (serum-free medium, SFM) to remove FBS contaminants. MEM/EBSS SFM was added and cells were cultured for 18 h.

Infection assays and sample preparation for LC-MS/MS analyses

The NPTr swine cells were infected with *M. hyopneumoniae* 7448, *M. hyopneumoniae* J or *M. flocculare* as described by Leal Zimmer et al. (2018). Each infection assay, and each control culture was independently performed three times (biological replicates). Cells were scratched from culture flasks and recovered in PBS pH 7.2. Detached cells were washed two times with PBS and resuspended in 200 µl of 0.1% RapiGest (Waters Corporation, MA, USA). Cell lysis was performed by sonication (Sonicator Q500, Qsonica) in ice, using 5 pulses (30% amplitude) of 30 s each and 1 min interval between pulses. The lysates were clarified by centrifugation (15,000 ×g for 30 min) at 4 °C. Sample quality was assessed by 12% SDS-PAGE. Quantitation was performed using the microBCA Protein Assay Kit (Thermo Fisher Scientific, IL, USA), and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, IL, USA). For LC-MS/MS, 50 µg of protein were reduced with 5mM of dithiothreitol (Thermo Fisher Scientific, IL, USA) at 60 °C for 30min, and alkylated with 15 mM of iodoacetamide (Bio-Rad, CA, USA) in the dark at room temperature for 30 min, and further digested with 1 µg of trypsin (Promega, WI, USA) overnight at 37°C. RapiGest SF Surfactant removal after digestion was carried out with 0.5% trifluoroacetic acid (TFA) (v:v). Resulting peptides were desalted in HLB cartridges (Waters Corporation, MA, USA) and eluted with 50% acetonitrile/0.1% TFA. Peptides were then dried using a Concentrator Plus (Eppendorf, DE), and kept at -20°C, until analysis.

LC-MS/MS for protein identification

Peptide samples from total cell lysates were reconstituted in a 0.1% formic acid (FA) in water solution (Thermo Fisher Scientific, IL, USA), and applied to an on-line LC-MS/MS system, including a HPLC NanoAcquity System (Waters Corporation, MA, USA), and an Orbitrap Elite™ Hybrid IonTrap-Orbitrap mass spectrometer (Thermo Fischer Scientific, IL, USA), as described in Paes et al. (2017). Each biological replicate was independently analyzed by LC-MS/MS three times (technical replicates).

LC-MS/MS data analysis

MS data was processed using the Mascot Software (Matrix Science, UK). Raw MS data files were processed using Mascot Distiller. The database search was performed against

local *Sus scrofa* databases containing the deduced amino acid sequences (with 48,617 sequences), available at NCBI (https://www.ncbi.nlm.nih.gov/protein). For protein identifications the search parameters included a fragment ion mass tolerance of 0.6 Da, peptide ion tolerance of 10 ppm. One missed cleavage of trypsin was allowed, carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine was included as variable modifications. Scaffold software version 4.4.1 (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 >95.0% probability as assigned by the Peptide Prophet algorithm (Sardana et al., 2008). The protein identifications were accepted if they could be established at >99% probability as assigned by the Protein Prophet algorithm (Hasan et al., 1999). Swine protein identifications were validated only when at least 2 corresponding peptides were detected. Any validated identified proteins were accepted only if they were detected in at least two of three biological replicates. The normalized spectral abundance factor (NSAF) (Zybailov et al., 2006) was calculated for each identified protein in Scaffold software and used to quantify relative differences in protein abundance between samples. Quantitative differences were statistically analyzed using the Student's t-test in GraphPad Prism 6 software. Volcano plot analysis, to demonstrate the magnitude, fold change (FC), and significance of quantitative differences, were performed in Scaffold. Proteins with Log_2 FC ≥ 0.5, and $p \leq 0.05$, were considered differentially represented between compared samples and numbered in the volcano plot. The heat map was performed with NSAF values normalized by Z-score calculation; hierarchical clustering was used as method of cluster analysis and the distance between proteins was calculated by Euclidean distance. The heat map analysis was performed in Heatmapper tool (Babicki et al., 2016), available at http://www.heatmapper.ca.

Bioinformatics analysis of protein function annotation and biochemical pathways

Online BLASTP searches were performed using BLAST2GO version 3.0 against the NCBInr database. GO mapping, InterProScan analysis and annotation were performed based on BlastP results (E-value $\leq 1.0 \times 10^{-3}$). Bar charts were generated using all available GO subcategories of level 2. The KAAS server (Moriya et al., 2007) was used to map KEGG biochemical pathways; the bi-directional best hit method was used to assign orthologues

(threshold of BLAST bit scores = 50). The representative gene data set for *Sus scrofa* was used as references in KAAS mapping.

RESULTS

SDS-PAGE profiling and LC-MS/MS identification of proteins in NPTr cells infected or not with *M. hyopneumoniae* 7448

Protein content and quality of three different samples (biological replicates) of NPTr cells infected with *M. hyopneumoniae* 7448 (named NPTr-Mh7448) and the corresponding non-infected control were initially assessed by 12% SDS-PAGE analysis. NPTr-Mh7448 and control samples showed a complex mixture of proteins ranging from 220 kDa to 30 kDa, and similar profiles were observed for all replicas in both samples (Figure S1).

NPTr-Mh7448 and control total protein extracts were analyzed by LC-MS/MS and the repertories of detected swine proteins were compared to each other for identification of proteins related to host-pathogen interaction. A summary of the LC-MS/MS results is presented in Figure 1, and the full lists of identified proteins and peptides are shown in Table S1A-B and Table S2A-B, respectively. Overall, 853 swine proteins species were detected, 695 in NPTr-Mh7448 samples, and 762 proteins in the control samples. Ninety-one (10.7%) and 158 (18.5%) proteins were exclusively detected in the NPTr-Mh7448 and control samples, respectively, while 604 proteins (70.8%) were shared between them.

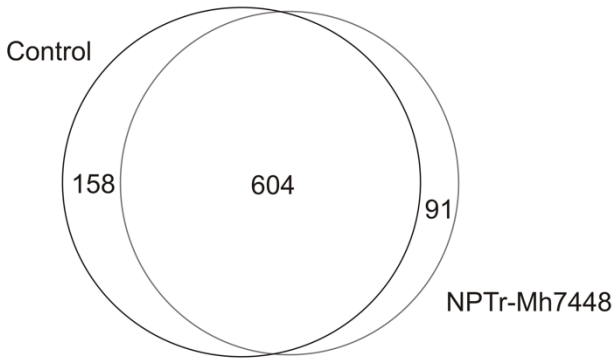

Figure 1. Overview of the proteins identified in the NPTr-Mh7448 and control samples. The numbers of proteins exclusively detected in each sample or shared between them are indicated in the diagram. Only proteins identified by at least two peptides and present in at least two biological replicates were considered for analysis.

A set of 541 proteins from the 604 shared between NPTr-Mh7448 and control samples did not present any statistically significant difference in abundance ($p \geq .05$ and FC $\geq$ 1.5 or ≤ 0.66) based on NSAF quantitative data. In this set, there are structural proteins (e.g. actin, filamin and tubulin), regulatory proteins (including 14-3-3 proteins, ras-related proteins), and proteins involved in basic cell functions, as replication, transcription and translation, including DNA replication factors, translation initiation and elongation factors, and several ribosomal proteins.

Differentially represented proteins in NPTr cells in response to *M. hyopneumoniae* 7448 infection

Differentially represented proteins, including those exclusively detected, overrepresented or underrepresented in NPTr-Mh7448 samples in comparison to the control ones were assessed in order to highlight the changes in NPTr cells in response to the infection with *M. hyopneumoniae* 7448. The repertoire of 91 NPTr-Mh7448 exclusive proteins (Table S1A) included histones and a histone binding protein, ribosomal proteins, proteins involved with danger signaling and cell death (annexin A11, protein S100-A11, apoptosis-inducing factor and apoptotic chromatin condensation inducer), and cytochrome c subunits (cytochrome c oxidase subunit 4 isoform 1 and cytochrome c oxidase subunit 7A2). Among

the 158 control-exclusive proteins (Table S1B) there were thioredoxin proteins, proteasome subunits, heterogeneous nuclear ribonucleoproteins, and translation initiation factors.

A volcano plot analysis was performed in order to investigate the magnitude of the difference in protein abundance between the repertoires of shared proteins with significantly different abundances between NPTr-Mh7448 and control samples. In this analysis (Figure 2), 65 were established as differentially abundant, being 38 overrepresented and 27 underrepresented in NPTr-Mh7448 samples. Among the NPTr-Mh7448 overrepresented proteins, there were stress proteins (heat shock protein 75 kDa, stress-70 protein, hypoxia up-regulated protein and annexin A4), and immunity-related proteins (interleukin enhancer binding factor 3 and stomatin-like protein 2). The proteins exclusively identified in NPTr-Mh7448 samples were merged with the differential proteins with FC ≥ 1.5 in a single set of overrepresented proteins for further functional analyses. The NPTr-Mh7448 underrepresented protein set (FC ≤ 0.66) included proteins involved with DNA replication and gene expression (DNA replication licensing factor MCM4, splicing factor 3A subunit 1, translation initiation factor 4A-I, and bifunctional glutamate/proline-tRNA ligase) and general metabolism (e.g. ATP citrate lyase, cAMP-dependent protein kinase type II-alpha regulatory subunit, and D-3-phosphoglycerate dehydrogenase).

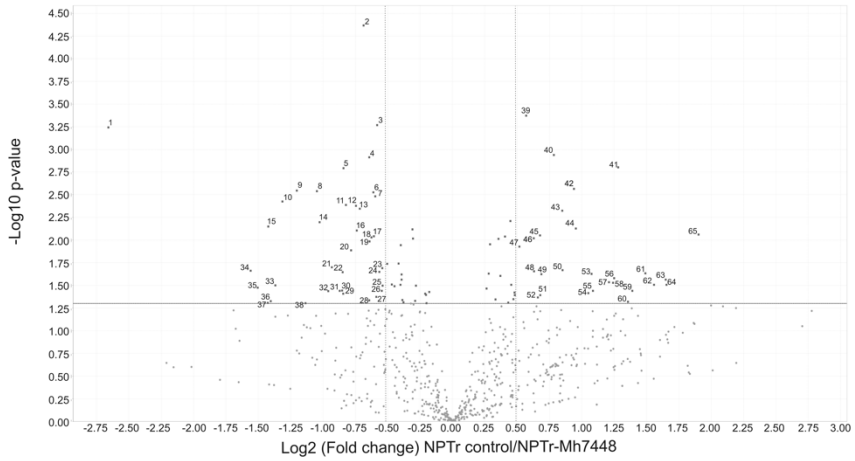

Figure 2. Volcano plot of shared NPTr-Mh7448 and control proteins showing significant differences between samples. Proteins with a *p* value < 0.05 are represented above the line and proteins with a fold-change (FC) > 1.5 (Log_2FoldChange 0.5) are identified by their numbering. Differential protein annotations are as follows: (1) ERO1-like protein alpha precursor; (2) stress-70 protein, mitochondrial; (3) interleukin enhancer binding factor 3, 90 kDa; (4) cytosol aminopeptidase; (5) protein disulfide-isomerase A4 precursor; (6) annexin

A4; (7) voltage-dependent anion-selective channel protein 1; (8) 78 kDa glucose-regulated protein; (9) ATP synthase subunit d, mitochondrial; (10) cytoskeleton-associated protein 4; (11) serine palmitoyltransferase 1; (12) histone H1.4-like; (13) citrate synthase, mitochondrial; (14) leucine-rich repeat-containing protein 59; (15) UDP-glucuronosyltransferase 1A6; (16) The Bowman-Birk type Inhibitor; (17) dihydrolipollysine-residue acetyltransferase; (18) glycine - tRNA ligase; (19) ras-related protein Rab-7a; (20) Ppk 98, a protein kinase; (21) LIM domain only protein 7-like; (22) neutral amino acid transporter B(0)-like; (23) heat shock protein 75 kDa, mitochondrial; (24) histone H2AX; (25) hypoxia up-regulated protein 1; (26) extended synaptotagmin-1; (27) DEAD-box helicase 21; (28) dolichyl-diphosphooligosaccharide protein glycosyltransferase subunit 1; (29) calnexin isoform X1; (30) stomatin-like protein 2; (31) ribosomal L1 domain containing 1; (32) NHP2-like protein; (33) gag protein; (34) nucleolar protein 56; (35) mitochondrial glutamate carrier 1; (36) 40S ribosomal protein S3a; (37) UDP-glucose glycoprotein glucosyltransferase 1; (38) keratin, type 1 cytoskeletal 18; (39) alpha-actinin-4; (40) calpain-2 catalytic subunit; (41) bifunctional glutamate/proline-tRNA ligase; (42) eukaryotic initiation factor 4A-1; (43) capine-1; (44) splicing factor 3A subunit 1; (45) hsc70 - interacting protein; (46) ras GTPase-activing-like protein IQGAP1; (47) heterogeneous nuclear ribonucleoprotein A2/B1; (48) ATP dependent 6-phosphofructokinase; (49) protein phosphatase 2A beta; (50) cAMP dependent protein kinase type II-alpha; (51) D-3-phosphoglycerate dehydrogenase; (52) serine-threonine kinase receptor-associated protein; (53) triosephosphate isomerase 1; (54) catenin alpha-1; (55) CD109 antigen; (56) talin-1; (57) DNA replication licensing factor MCM4; (58) spermine synthase; (59) peroxiredoxin-6; (60) prolow-density lipoprotein receptor-related protein-1; (61) 26S proteasome non-ATPase regulatory subunit 11; (62) cytoplasmic dynein 1; (63) ATP citrate lyase; (64) caprin-1; (65) coronin-1B. Proteins without significant differences in abundance between samples are shown as black dots.

Functional categorization of differential NPTr-Mh7448 proteins

To get a functional overview of the 129 overrepresented and 27 underrepresented NPTr-Mh7448 differential proteins, they were separately categorized based on gene ontology (GO) annotations and KEGG biochemical pathways. GO term assignment was possible for 95.5% of the differential proteins (148 out of 155 proteins), which were categorized according to the three main GO categories: ‘biological process’, ‘molecular function’ and ‘cellular component’ (Figure 3 and Table S3). GO classifications set at level 2 resulted in the assignment of the differential NPTr-Mh7448 proteins to 29 and 33 subcategories for over- and underrepresented ones, respectively. Most subcategories in the three ‘biological process’, ‘molecular function’ and ‘cellular component’ categories were shared by both NPTr-Mh7448 overrepresented and underrepresented protein sets. The ‘metabolic process’, ‘response to stimulus’, ‘signaling’, ‘immune system process’; ‘binding’, ‘catalytic activity’ and ‘structural molecule activity’; and ‘organelle’, ‘membrane’, ‘macromolecular complex’ are examples of

shared subcategories. On the other hand, some subcategories were found as exclusive for one or another set of proteins. ‘Molecular function regulator’, a ‘molecular function’ subcategory, was the only exclusive for the overrepresented protein set, and, interestingly, this subcategory includes, at level 4 of GO annotation, three subcategories involved with cell death (‘cell death’, ‘negative regulation of cell death’, and ‘regulation of cell death’) (Table S3C and D). For the underrepresented protein set, in turn, four level 2 subcategories were exclusively, namely ‘biological adhesion’, ‘locomotion’, ‘transporter activity supramolecular complex’ and ‘cell junction’ subcategories were found as exclusive at level 2.

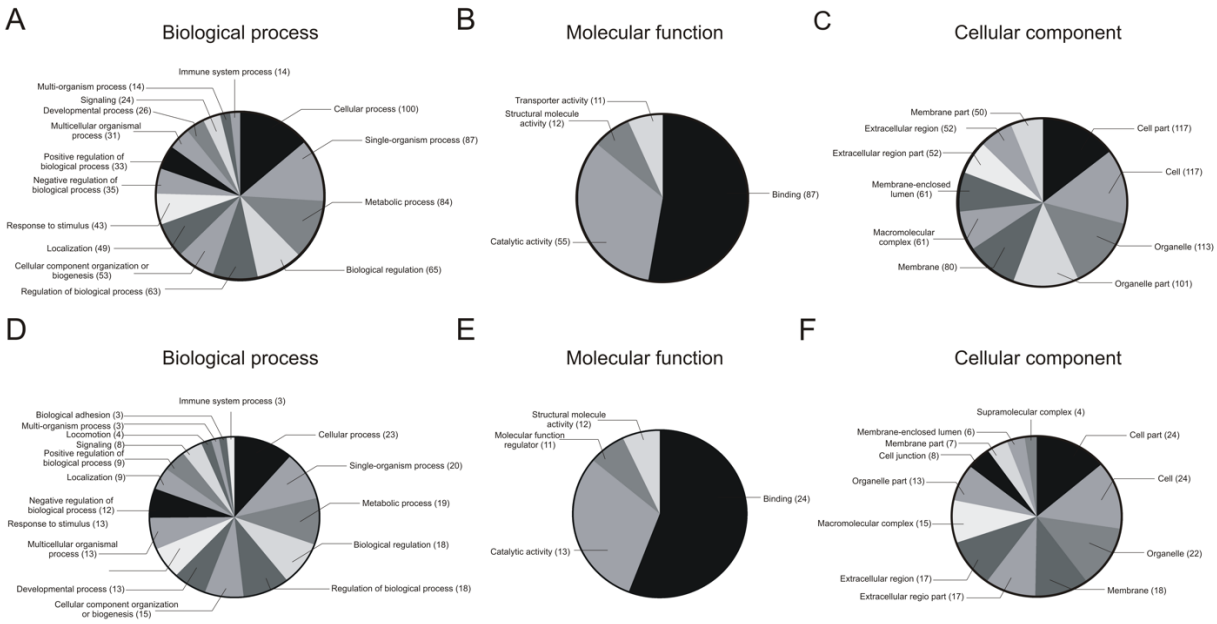

Figure 3. Gene ontology (GO) profile of differentially represented proteins identified in the NPTr-Mh7448 sample. GO distribution of overrepresented (A to C) and underrepresented (D to F) proteins in the functional annotations ‘biological process’, ‘molecular function’, and ‘cellular component’ until the second level of complexity. The results of BlastP searches against the NCBInr database were used for GO mapping and annotation. Some of the proteins were assigned to more than one functional category.

At least one KEGG biochemical pathway code was successfully assigned to 142 of the 155 NPTr-Mh7448 differential proteins, comprehending 117 overrepresented proteins (Table S4A) and 25 underrepresented ones (Table S4B). The 155 differential proteins were assigned to a total of 183 unique pathways, 163 for overrepresented protein and 63 for underrepresented ones (Table S4C and D, respectively). Forty-three of the 183 unique

pathways are common to both over and underrepresented protein sets, while 120 (73.6%) and 20 (31.7%) of them were found only for NPTr-Mh7448 overrepresented or underrepresented proteins, respectively. The top 10 NPTr-Mh7448 KEGG assignments for overrepresented and underrepresented, shown in Table 1, revealed marked differences between these protein sets, sharing only the top four pathways, related to basic metabolism, and differing in the other 6. The 6 top KEGG pathways in the overrepresented NPTr-Mh7448 set were 'protein processing in endoplasmic reticulum', 'Parkinson's disease', 'Huntington's disease'. 'RNA transport', 'oxidative phosphorilation', and 'phagosome'. Moreover, other infection-related biochemical pathways, as 'PI3K-Akt signaling pathway', 'MAPK signaling pathway', 'IL-17 signaling pathway', 'TGF-beta signaling pathway', 'Fc gamma R-mediated phagocytosis', 'antigen processing and presentation', and 'apoptosis' were also exclusive among NPTr-Mh7448 overrepresented proteins. On the other hand, the 6 top exclusive KEGG biochemical pathways in the underrepresented NPTr-Mh7448 set were 'microbial metabolism in diverse environments', 'biosynthesis of amino acids', 'focal adhesion', 'adherens junction', 'methane metabolism' and 'leukocyte transendothelial migration'.

Table 1. Top 10 most represented KEGG pathways in differentially abundant proteins detected in NPTr-Mh7448 samples (overrepresented and underrepresented). The numbers of proteins assigned to each biochemical pathway are indicated. Bi-directional best hit method to assign the orthologs (threshold of BLAST bit scores = 50) were the parameters used. The representative gene data set for *Sus scrofa* was used as references in KAAS mapping.

Overrepresented		**Underrepresented**	
Pathway name	Number of the represented proteins in NPTr-Mh7448 samples	Pathway name	Number of the represented proteins in the control samples
ko01100 Metabolic pathways	20	ko01100 Metabolic pathways	7
ko04141 Protein processing in endoplasmic reticulum	13	ko01110 Biosynthesis of secondary metabolites	5
ko01110 Biosynthesis of secondary metabolites	9	ko01120 Microbial metabolism in diverse environments	5
ko05012 Parkinson's disease	7	ko01130 Biosynthesis of antibiotics	4
ko05016 Huntington's disease	7	ko01200 Carbon metabolism	3
ko03013 RNA transport	6	ko01230 Biosynthesis of amino acids	3
ko00190 Oxidative phosphorylation	5	ko04510 Focal adhesion	3
ko01130 Biosynthesis of antibiotics	5	ko04520 Adherens junction	3
ko01200 Carbon metabolism	5	ko00680 Methane metabolism	2
ko04145 Phagosome	5	ko04670 Leukocyte transendothelial migration	2

Comparative analyses of infection-related proteins between NPTr cells infected with pathogenic and non-pathogenic mycoplasmas

LC-MS/MS analyses were also performed with NPTr cells infected with the non-pathogenic *M. hyopneumoniae* J strain (NPTr-MhJ) and the genetically related commensal *M. flocculare* (NPTr-Mf), in the same conditions used for the assays with *M. hyopneumoniae* 7448. Totals of 774 and 704 swine proteins were identified in the NPTr-MhJ and NPTr-Mf samples, respectively (Table S1C and D). The set of NPTr-Mh7448 differential proteins assigned the GO 'immune system' and 'response to stimulus' subcategories' (57 proteins) was then selected for comparison to the corresponding sets of proteins from NPtr-MhJ and NPTr-Mf samples.

From the 57 NPTr-Mh7448 infection-related proteins, 18 and 23 were also detected in NPTr-MhJ and NPTr-Mf samples, respectively (Table S5). Eleven and 16 of these proteins shared with NPTr-MhJ and NPTr-Mf, respectively, showed significant differences (p value < 0.05) when compared with NPTr-Mh7448. Interestingly, with few exceptions, most proteins that had significant abundance differences in the NPTr-Mh7448/NPTr-MhJ and NPTr-Mh7448/NPTr-Mf comparisons, showed the same pattern (over or underrepresentation) found in the NPTr-Mh7448/control comparison (Figure 4). Moreover, most of these proteins were overrepresented in NPTr-Mh7448, and are involved with stress and danger response, as 78 kDa glucose-regulated protein, heat shock protein 75 kDa, stress-70 protein, mitochondrial, histone H2AX and interleukin enhancer binding factor 3. These results demonstrate that at least some infection-related proteins had their abundance altered exclusively in response to pathogenic *M. hyopneumoniae* 7448 infection, and likely contributing to disease outcome at the cellular level.

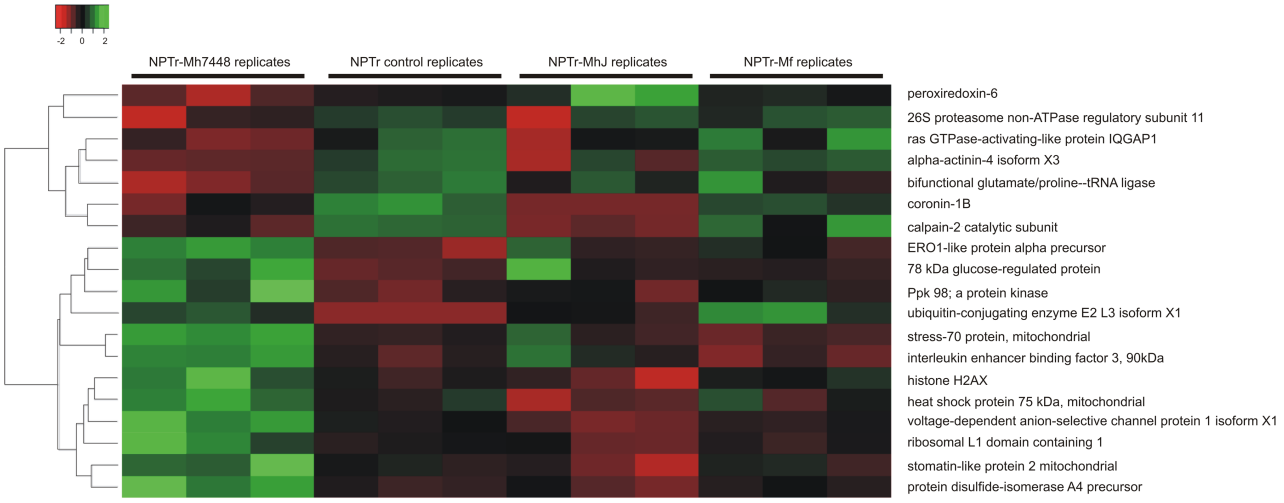

Figure 4. Heat map of differentially abundant proteins among NPTr-Mh7448, control, NPTr-MhJ and NPTr-Mf samples. All shared proteins showing statistically significant abundance differences between samples ($p < 0.05$) are represented (green: higher abundances; red: lower abundances), and their protein names are shown on the left. NSAF values were used to quantify relative differences in protein abundance among shared proteins and heat map was plotted using Euclidian distance and Z-score.

DISCUSSION

Swine response against *M. hyopneumoniae* has been related to lung lesions observed during PEP development. There is infiltration of both neutrophils and mononuclear cells in the lamina propria of the respiratory tract upon *M. hyopneumoniae* adherence, which increases the local production of pro-inflammatory cytokines (Livingston et al., 1972; Choi et al., 2006; Hwang et al., 2011). Moreover, it has been demonstrated that *M. hyopneumoniae* induces swine cell death (Bai et al., 2015; Paes, Virginio, et al., 2017; Ferrarini et al., 2018), as the presence of *M. hyopneumoniae* itself or some of its byproducts trigger swine cell mechanisms from specific cytokine production to apoptosis. In order to identify cell pathways modulated in response to *M. hyopneumoniae* infection, in this work, a LC-MS/MS proteomic approach was performed to analyze total protein lysates of a swine trachea cell line (NPTr cells) infected with *M. hyopneumoniae* 7448.

Protein samples from total swine cell lysates showed high reproducibility among biological replicates, which was initially evidenced by their similar electrophoretic profiles, and later confirmed by the LC-MS/MS results. Comparison of electrophoretic profiles from NPTr-Mh7448 and NPTr non-infected control cells showed just slight differences, which was expected due to the complexity of the total cell lysate visualized by SDS-PAGE. On the other hand, in the comprehensive survey of the NPTr cell proteome infected or not with *M. hyopneumoniae* 7448, it was possible to identify and quantify a total of 853 swine proteins species by a highly sensitive LC-MS/MS. Overall, 156 proteins in *M. hyopneumoniae*-infected NPTr cells were identified as differentially represented (qualitatively or quantitatively) in comparison to the control, which provides evidences of swine cell responses against *M. hyopneumoniae*. Changes in gene expression of porcine alveolar macrophages infected with *M. hyopneumoniae* have already been demonstrated (Bin et al., 2014), but in this work, a epithelial cell line was used to identified proteins involved in *M. hyopneumoniae* response. Moreover, we performed a proteomic analysis, which is result of post-transcriptional and post-translation modifications and can elucidate other modifications upon *M. hyopneumoniae* infection than transcriptomic studies.

Among KEGG pathways, the 'Phagosome' one was exclusive for NPTr infected cells and suggests phagocytosis as a cell mechanism to eliminate *M. hyopneumoniae*. This pathway was also identified as one of the most significant pathways in porcine alveolar macrophages infected with *M. hyopneumoniae* (Bin et al., 2014). Interaction with surface accessible actin on the epithelial cells has been already demonstrated (Raymond et al., 2018) and may cause cytoskeletal rearrangements allowing the organism to be phagocytosed (Maes et al., 2017). KEGG pathways involved with immune systems were also found exclusively among overrepresented proteins in NPTr infected cells. , as PI3K-Akt signaling pathway', 'IL-17 signaling pathway', 'TGF-beta signaling pathway', 'Fc gamma R-mediated phagocytosis', and 'antigen processing and presentation'. These results suggest other pathways involved in *M. hyopneumoniae* response to be explored with exception of the NFκB and MAPK signaling pathway already described as affected during *M. hyopneumoniae* infection (Hwang et al., 2011; Ni et al., 2015).

Functional GO annotation analyses also highlighted that differentially abundant swine proteins identified by our study may be involved with *M. hyopneumoniae* pathogenesis. One

third of the 156 proteins were assigned to 'biological process' subcategories related to infection processes, as 'immune system process' and/or 'response to stimulus'. The abundance of differentially represented proteins in response to *M. hyopneumoniae* and assigned in the 'immune system process' and 'response to stimulus' functional subcategories were evaluated in NPTr cells infected with non-pathogenic mycoplasmas *M. hyopneumoniae* J or *M. flocculare*. These analyses provided a set of proteins that are differentially represented exclusively in response to pathogenic *M. hyopneumoniae* 7448, suggesting their involvement in the *M. hyopneumoniae* pathogenesis. Among the proteins exclusively detected in the NPTr-Mh7448 samples and not in the control and NPTr-MhJ or NPTr-Mf, and those with significant abundance differences, there are several proteins related to apoptosis, stress and immune system.

Since *M. hyopneumoniae* induces Ca^{2+} release from intracellular endoplasmic reticulum stores in porcine tracheal epithelial cells (Park et al., 2002), proteins identified here may be related to this mechanism. The proteins 78 kDa glucose-regulated, Ppk 98 and stomatin-like protein 2 were found as overrepresented in NPTr-Mh7448 sample and in low abundance in NPTr-MhJ, NPTr-Mf or control samples. These are endoplasmic reticulum associated proteins and their expression can be modulated by perturbation in intracellular homeostasis. Voltage-dependent anion-selective channel protein 1 (VDAC1), in turn, is a Ca^{2+} mediator transporter present in the outer mitochondrial membrane (OMM) and its overexpression was found to induce apoptotic cell death (Shoshan-Barmatz et al., 2015). VDAC1 was also exclusively overrepresented in the NPTr-Mh7448 sample, and may be mediating dysregulation of calcium homeostasis, leading to the swine cell apoptosis observed during *M. hyopneumoniae* 7448 infection. Indeed, the two apoptosis-inducing factor mitochondrion-associated 1, apoptotic chromatin condensation inducer in the nucleus, and annexin A11 are apoptosis inducers and were found exclusively in the NPTr-Mh7448 sample.

Endoplasmic reticulum (ER) stress also seems to be present in NPTr cells infected with *M. hyopneumoniae* 7448, as suggested by exclusivity or overrepresentation of endoplasmic reticulum chaperones, as 78 kDa glucose-regulated protein (also known as GRP78), ERO-like protein alpha precursor, disulfide-isomerase A4 and ubiquitin-conjugating enzyme E2 L3. ER stress can be induce by a wide range of cellular perturbations, including loss of calcium homeostasis (Hetz et al., 2015). Irremediable ER stress can turns signaling towards

a ‘terminal unfolded protein response (UPR)’ that drives cells into apoptosis. Many of the pro-death signals emerging from UPR response regulate the canonical mitochondrial apoptotic pathway. This pathway is initiated when pro-apoptotic mitochondrial proteins, such as cytochrome c, are actively released into the cytoplasm to trigger the proteolytic activation of effector caspases such as caspase-3. In this line, we also exclusively identified cytochrome c subunits in the NPTr-Mh7448 samples, suggesting that cell apoptosis observed in PEP may be trigger by ER stress response. Apoptosis by ER stress has also been related to infection with other bacteria (Lim et al., 2011; Oh et al., 2018).

In summary, our results showed that the proteome of a swine respiratory epithelial cell is significantly altered in response to infection with a pathogenic *M. hyopneumoniae* strain including immune and stress-related proteins. A comparative analysis of expression of these proteins in NPTr cells infected with non-pathogenic mycoplasmas, as *M. hyopneumoniae* J or *M. flocculare* reveals proteins whose abundance is altered exclusively by pathogenic *M. hyopneumoniae* 7448 infection. Our data shed light on possible swine cell processes contributing to *M. hyopneumoniae* pathogenesis, suggesting the involvement of calcium homeostasis and ER stress with *M. hyopneumoniae*-induced apoptosis events in swine cells.

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4.3 Análise proteômica de vesículas extracelulares secretadas por uma linhagem celular de traqueia suína infectada com a uma cepa patogênica de *M. hyopneumoniae*.

Materiais e Métodos

Cultura de células de *M. hyopneumoniae* e da linhagem celular NPTr

M. hyopneumoniae 7448 foi isolado de um suíno naturalmente infectado de Lindóia do Sul (SC, Brazil) e sua patogenicidade foi experimentalmente demonstrada (Vasconcelos et al., 2005). *M. hyopneumoniae* foi cultivado em meio Friis (Friis, 1971) contendo 25% de soro fetal bovino (SFB) em 25 ml a 37ºC por 48 h com agitação (Leal et al., 2016).

A linhagem celular Newborn Porcine Trachea (NPTr) (Ferrari et al., 2003) foi adquirida do Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy) e cultivada em meio MEM/EBSS suplementado com 10% SFB a 37°C em uma atmosfera umidificada contendo 5% CO^2.

Ensaios de infecção

Os ensaios de infecção entre a linhagem celular NPTr e *M. hyopneumoniae* 7448 foram realizados como descrito em Leal Zimmer et al., (2018). Cultivos de células NPTr e de *M. hyopneumoniae* 7448 foram utilizados como controles. Cada ensaio de infecção e cada cultura controle foi realizado 4 vezes.

Isolamento de vesículas extracelulares e preparação das amostras para LC-MS/MS

O sobrenadante dos ensaios de infecção e das culturas controle foram misturados para o isolamento das vesículas extracelulares, resultando em três amostras diferentes: ensaio de infecção (NPTr-Mh7448), cultura controle NPTr, cultura controle *M. hyopneumoniae* 7448. O total de sobrenadante para cada amostra foi 60 ml. O isolamento das vesículas extracelulares foi realizado de acordo com Théry et al. (2006). O precipitado final contendo as vesículas extracelulares foi ressuspendido em 50 µl de RapiGest SF 0,1% (Waters Corporation, MA, USA). A qualidade das amostras foi verificada por SDS-PAGE 12% e a quantificação das proteínas foi realizada utilizando o kit microBCA Protein Assay (Thermo Fisher Scientific, IL, USA) e o espectrofotômetro NanoDrop 2000 (Thermo Fisher Scientific, IL, USA). Para as análises de LC-MS/MS 2 µg de proteínas foram reduzidas com 5mM de

dithiotreitol (Thermo Fisher Scientific, IL, USA) a 60°C por 30 min e alquilado com 15 mM de iodoacetamida (Bio-Rad, CA, USA) a temperatura ambiente por 30 min em ambiente escuro. As proteínas então foram digeridas com 1 mg de tripsina (Promega, WI, USA), a 37°C durante 16 h. O surfactante RapiGest SF foi removido com ácido trifluoroacético (TFA) 0,5% (v:v). Os peptídeos resultantes foram dessalinizados em colunas HLB (Waters Corporation, MA, USA) e eluídos em acetonitrila 50%/TFA 0,1%. Os peptídeos foram concentrados utilizando o Concentrator Plus (Eppendorf, DE) e armazenados a -20°C até a análise de LC-MS/MS.

Análise de LC-MS/MS das proteínas extraídas das amostras de vesículas extracelulares

As amostras de peptídeos foram reconstituídas em 0,1% de ácido fórmico (FA) e aplicadas em sistema de LC-MS/MS incluindo um HPLC NanoAcquity System (Waters Corporation, MA, USA), e um Orbitrap Elite™ Hybrid IonTrap-Orbitrap mass spectrometer (Thermo Fischer Scientific, IL, USA), como descrito por Leal Zimmer et al., 2018. Cada pool foi analisado por LC-MS/MS independentemente três vezes. Os dados de MS no formato .raw foram processados utilizando Mascot Distiller (Matrix Science, UK). A pesquisa da base de dados foi realizada contra um banco contendo as sequencias de aminoácidos das proteínas de *Sus scrofa* e de *M. hyopneumoniae* 7448 (com 48.617 e 819 sequências, respectivamente) disponíveis no NCBI (https://www.ncbi.nlm.nih.gov/protein). Para identificação das proteínas suínas e de mycoplasma, os parâmetros incluídos foram 0.6 Da para a tolerância de massa do íon fragmento e 10 ppm para a tolerância do íon de peptídeo. Para as proteínas suínas, uma clivagem perdida foi permitida para tripsina, carbamidometilação da cisteína foi especificada como modificação fixa e oxidação da metionina foi incluída como modificação variável. Para as proteínas de *M. hyopneumoniae* 7448, a perda de até três clivagens foi permitida e carbamidometilação da cisteína foi incluída como modificação fixa. As modificação variáveis incluíram oxidação da metionina, acetilação da lisina e das extremidades N-terminais das proteínas, e fosforilação da tirosina e serina/treonina (Chen et al., 2016, Lluch-Senar et al., 2016). A identificação dos peptídeos foi aceita quando alcançaram ≥ 95% de probabilidade conforme o algoritmo Peptide Prophet (Sardana et al., 2008) e a identificação das proteínas foi aceita quando alcançaram ≥ 99%de probabilidade conforme o algoritmo Protein Prophet (Hasan et al., 1999). As proteínas suínas

e de *M. hyopneumoniae* foram validades se ao menos 2 peptídeos correspondentes foram detectados. As proteínas validadas foram aceitas somente quando detectadas em ao menos 2 das três corridas de LC-MS/MS. O fator de abundancia espectral normalizado (NSAF, do inglês *normalized spectral abundance factor*) (Zybailov et al., 2006) foi calculado para cada proteína identificada no software Scaffold e usado para quantificar as diferenças relativas na abundância proteica entre as amostras. Diferenças significativas foram estatisticamente analisadas utilizando Student's t-test no software Scaffold. Proteínas com valor $p \leq 0,05$ foram consideradas diferencialmente abundantes.

Análise de bioinformática das funções proteicas e localização celular

As proteínas suínas foram submetidas a sobre-representação hierárquica de GO utilizando o plugin BiNGO 2.3 disponível no software Cytoscape 2.6.3 (Maere et al., 2005). Os arquivos de ontologia foram retirados do banco de dados GO (http://www.geneontology.org/) e a anotação das proteínas de suíno foi retirada do Uniprot (http://www.uniprot.org). A sobre-representação hipergeométrica foi realizada com $p \leq 0,05$ de significância, com a correção de Benjamini-Hochberg FDR. Os termos de GO foram sumarizados utilizando o site REVIGO (Supek et al., 2011), com similaridade calculada através de SimRel (similaridade permitida = 0,7).

Predições *in silico* das vias de secreção das proteínas suínas e de *M. hyopneumoniae* 7448 identificadas foram realizadas utilizando a pipeline descrita por Monteiro et al. (2017). A secreção dependente de peptídeo sinal foi predita utilizando SignalP 4.1 (Petersen et al., 2011), Phobius (Käll et al., 2004), e PrediSI (Hiller et al., 2004). A secreção independente de peptídeo-sinal foi realizada com SecretomeP 2.0 (Bendtsen et al., 2004). Proteínas detectadas sem predição de via de secreção foram analisadas quanto a presença de ortólogos no banco de dados de vesículas extracelulares Vesiclepedia (Kalra et al., 2012).

Resultados

Identificação das proteínas suínas presentes nas vesículas extracelulares

As amostras de vesículas extracelulares foram analisadas primeiramente por SDS-PAGE (Figure 1). O perfil eletroforético das amostras NPTr-Mh7448 e NPTr apresentou

padrão de bandas similar, mas com algumas diferenças qualitativas e quantitativas. Na amostra de *M. hyopneumoniae* 7448 não foi possível observar nenhuma banda de proteína.

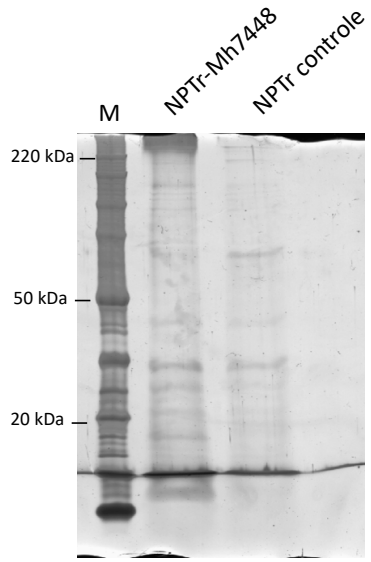

Figura 1. Perfil eletroforético das amostras de vesículas extracelulares de NPTr-Mh7448 e NPTr controle. Uma quantidade de 2,5 μg de cada amostra foi aplicada em cada canaleta. M: marcadores de massa molecular (kDa); SDS-PAGE 12% e coloração de nitrato de prata 2%.

As proteínas suínas presentes nas vesículas extracelulares das condições ensaio de infecção (NPTr-Mh7448) e das culturas-controle (NPTr e *M. hyopneumoniae* 7448) foram identificadas através da análise por LC-MS/MS. Um total de 245, 98 e 3 proteínas suínas foram identificadas nas amostras de NPTr-Mh7448, NPTr e *M. hyopneumoniae* 7448, respectivamente. A identificação e quantificação das proteínas e peptídeos estão apresentados nas tabelas suplementares S1 e S2, respectivamente. A taxa de falsa descoberta (FDR, do inglês *false discovery rate*) para as proteínas e peptídeos foi zero, validando os resultados de MS/MS. A Figura 2 apresenta uma visão geral das proteínas identificadas exclusivamente e compartilhadas em cada amostra. As amostras NPTr-Mh7448 e NPTr apresentaram 155 e 12 proteínas suínas exclusivas, respectivamente. Ambas amostras compartilharam 83 proteínas suínas. As 3 proteínas suínas identificadas na amostra *M. hyopneumoniae* 7448 (Tabela S1C) foram compartilhadas com as outras duas amostras (NPTr-Mh7448 e NPTr) e consideradas como contaminantes.

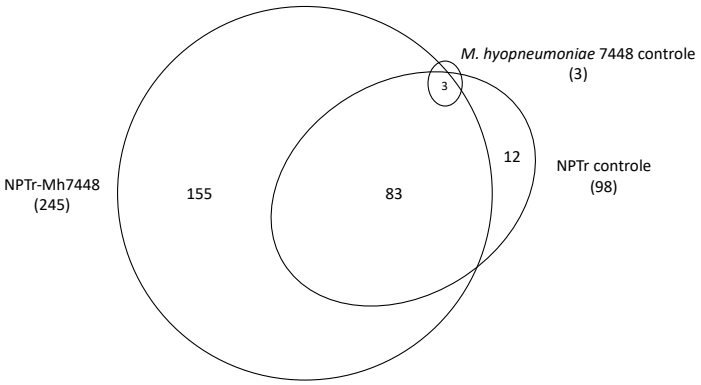

Figura 2. Proteínas suínas compartilhadas e exclusivas de cada uma das amostras (NPTr-Mh7448, NPTr ou *M. hyopneumoniae* 7448). O número de proteínas exclusivas e compartilhadas está demonstrado dentro dos círculos e o número total de proteínas está demonstrado fora dos círculos para cada amostra.

Considerando as 83 proteínas compartilhadas entre as amostras NPTr-Mh7448 e NPTr, 21 proteínas suínas apresentaram abundância com diferenças significativas (valor de $p \leq 0,05$) (Tabela S3). Apenas duas proteínas, histone H3-like e tubulin beta chain, apresentaram abundância maior na amostra NPTr-Mh7448. Entre as 19 proteínas suínas que apresentaram maior abundância na amostra NPTr, incluem heat shock 70kDa protein 8, annexin A8 e CD109 antigen. Portanto, o universo de proteínas diferencialmente abundantes para NPTr-Mh7448 consiste em 157 proteínas suínas (155 exclusivamente detectadas mais 2 com diferenças significativas em abundância) e para NPTr consiste em 31 proteínas suínas (12 exclusivamente detectadas mais 19 com diferenças significativas em abundância). Esses universos de proteínas diferencialmente representados foram utilizados nas análises de enriquecimento funcional a serem descritos abaixo.

Classificação funcional das proteínas suínas identificadas nas amostras de vesículas extracelulares

As proteínas suínas diferencialmente representadas das amostras de NPTr-Mh7448 (157 proteínas) e das amostras NPTr (31 proteínas) foram funcionalmente analisadas de acordo com análise de enriquecimento de GO (Tabela S4) nas categorias componente celular (CC), função molecular (FM) e processos biológicos (PB). Foram encontradas inferências funcionais para 114 proteínas da amostra NPTr-Mh7448 e para 16 proteínas da amostra NPTr.

Na categoria CC, para ambos os grupos de proteínas diferencialmente representadas (NPT-Mh7448 e NPTr), as subcategorias ‘vesicle’ e ‘extracellular membrane-bounded organelle’ estiverem entre as mais enriquecidas. As subcategorias ‘organelle’ e ‘cytoplasm’ também foram mais enriquecidas para NPTr-Mh7448. Na categoria FM, apenas 6 subcategorias foram encontradas para as proteínas de NPTr controle e todas também estão presentes entre as subcategorias encontradas para as proteínas NPTr-Mh7448. No entanto, as proteínas de NPTr-Mh7448 apresentaram um conjunto maior e mais diverso de subcategorias, demonstrando uma maior complexidade das funções moleculares desempenhadas pelas proteínas diferencialmente representadas de NPTr-Mh7448. Por fim, na categoria PB, várias subcategorias envolvidas com a infecção por *M. hyopneumoniae* 7448 foram exclusivamente encontradas nas proteínas da amostra NPTr-Mh7448 como por exemplo, ‘immune system process’, ‘cell death’, ‘phagocytosis’, ‘oxidation-reduction process’e ‘response to endoplasmic reticulum stress’. Por outro lado, as subcategorias exclusivamente encontradas nas proteínas da amostra NPTr controle envolvem funções gerais das células, como por exemplo, ‘regulation of metal ion transport’, ‘cytoskeleton organization’ e ‘regulation of signal transduction’.

Identificação das proteínas de *M. hyopneumoniae* 7448 presentes nas vesículas extracelulares

As proteínas de *M. hyopneumoniae* 7448 foram identificadas somente nas vesículas extracelulares da condição ensaio de infecção (NPTr-Mh7448) através da analise por LC-MS/MS (Tabela 1). Para a validação dos dados de MS/MS, o FDR foi estabelecido em 1% e apenas proteínas que apresentaram no mínimo 4 peptídeos correspondentes foram validadas. Um total de 22 proteínas de *M. hyopneumoniae* 7448 foram identificadas e validadas, sendo 18 fatores de virulência descritos na literatura (Paes et al., 2018). A identificação e quantificação dos peptídeos estão apresentados na tabela suplementar S5. Nenhuma proteína de *M. hyopneumoniae* foi identificada nas amostras-controle de *M. hyopneumoniae* 7448 e de células NPTr.

Tabela 1. Proteínas de *M. hyopneumoniae* 7448 identificadas por LC-MS/MS na amostra NPTr-Mh7448.
As proteínas em negrito são identificadas como fatores de virulência em *M. hyopneumoniae*.

Nome da proteína	Número de acesso	Peso molecular da proteína (Da)	Contagem exclusiva de peptídeos únicos	Contagem exclusiva de espectros únicos	Contagem exclusive de espectros	Porcentagem total de espectros	Porcentagem de cobertura da sequência
chaperone protein dnaK - heat shock protein 70	AAZ53444.1	65621.7	9	9	11	0.07%	22%
elongation factor EF-G	AAZ53452.2	77410.3	4	4	4	0.02%	8%
NADH oxidase	AAZ53459.1	51401.6	4	5	5	0.03%	13%
thiol peroxidase	AAZ53473.2	18483.8	4	4	5	0.03%	34%
pyruvate dehydrogenase E1-alpha subunit	AAZ53490.1	42325.4	4	4	4	0.03%	20%
pyruvate dehydrogenase	AAZ53491.1	36735.2	7	7	10	0.06%	37%
aminopeptidase	AAZ53504.1	39257.8	5	5	5	0.03%	21%
protein P97 - copy 1	AAZ53572.1	122904.2	18	19	24	0.14%	22%
protein P102 - copy 1	AAZ53573.1	102338.1	8	8	10	0.06%	13%
conserved hypothetical protein	AAZ53590.2	235653.5	6	6	6	0.03%	4%
periplasmic sugar-binding protein	AAZ53608.1	43911	5	5	5	0.03%	18%

thioredoxin	AAZ53755.1	12650.7	4	4	5	0.03%	49%
ATP synthase alpha chain	AAZ53845.1	57643.8	5	5	6	0.03%	12%
putative p216 surface protein	AAZ53862.1	216630	27	31	36	0.21%	21%
p76 membrane protein precursor	AAZ53863.1	159781.7	16	16	18	0.11%	16%
dihydrolipoamide dehydrogenase	AAZ53873.1	67066	4	4	6	0.03%	10%
46K surface antigen precursor	AAZ53879.1	45733.8	7	9	14	0.08%	27%
elongation factor EF-Tu	AAZ53889.1	44095.1	9	11	16	0.10%	30%
putative ATP binding protein	AAZ53955.2	124158.6	5	5	5	0.03%	5%
prolipoprotein p65	AAZ54018.1	71021.4	7	7	8	0.05%	14%
hypothetical protein MHP7448_0662	AAZ54024.2	136223.7	12	12	15	0.09%	15%
adhesin like-protein P146	AAZ54025.1	148891.5	16	17	19	0.11%	20%

Vias de secreção preditas para as proteínas de suíno e de *M. hyopneumoniae* identificadas nas vesículas extracelulares

Predições *in silico* foram realizadas para identificar as vias de secreção das proteínas suínas e de *M. hyopneumoniae* 7448 detectadas nas vesículas extracelulares de NPTr-Mh7448, nos controles NPTr e *M. hyopneumoniae* 7448 (Tabela S6). Entre as proteínas suínas, mais de um terço apresentou uma via de secreção predita. Para as 245 proteínas

identificadas na amostra NPTr-Mh7448, 53, 52 e 62 proteínas suínas foram preditas como sendo secretadas pela via clássica, não-clássica e por vesículas extracelulares, respectivamente (Tabela S6A). Para as 98 proteínas identificadas na amostra NPTr, 15, 15 e 25 proteínas suínas foram preditas como sendo secretadas pela via clássica, não-clássica e por vesículas extracelulares, respectivamente (Tabela S6B). Já para as 22 proteínas de *M. hyopneumoniae* 7448 identificadas na amostra NPTr-Mh7448, 7 e 4 foram preditas como sendo secretadas pela via clássica e não-clássica, respectivamente (Tabela S6C). Como não há um banco de dados de proteínas secretadas através vesículas extracelulares por bactérias, a predição melhor embasada por essa via não pode ser realizada.

5. DISCUSSÃO GERAL

A elucidação de mecanismos moleculares envolvidos em interações patógeno-hospedeiro é importante para o desenvolvimento de novas estratégias para o controle de doenças infecciosas (Khabbaz et al., 2014). Em qualquer situação, as intricadas interações entre um patógeno e a respectiva espécie hospedeira podem envolver centenas de proteínas, sendo que a caracterização de determinantes de virulência de patógenos e de seus alvos de interação no hospedeiro tem sido o principal foco das pesquisas relacionadas a doenças infecciosas (Yang et al., 2015). Apesar disso, muitos dos mecanismos que governam os vários estágios das infecções por diferentes patógenos ainda são pouco compreendidos, especialmente devido ao fato de serem mediados por múltiplos fatores.

As abordagens proteômicas utilizadas no presente estudo permitiram identificar determinantes suínos da PES e vias de resposta de células suínas à infecção com *M. hyopneumoniae*. Com o objetivo de identificar o maior número possível de vias celulares afetadas pela infecção, diferentes frações proteicas de células suínas foram analisadas, incluindo proteínas intracelulares, proteínas secretadas totais e proteínas secretadas por vesículas extracelulares. Além disso, as proteínas secretadas totais e as proteínas secretadas por vesículas extracelulares de *M. hyopneumoniae* 7448, *M. hyopneumoniae* J e *M. flocculare* também foram detectadas, identificadas e analisadas. A identificação das proteínas com abundância diferencial de uma linhagem celular de traqueia suína (NPTr) durante a infecção com micoplasmas patogênicos e não-patogênicos do trato respiratório suíno (*M. hyopneumoniae* 7448, *M. hyopneumoniae* J e *M. flocculare*) descritas aqui geraram informações importantes tanto sobre a resposta do hospedeiro à presença das linhagens de micoplasmas, como sobre as proteínas das mesmas envolvidas na interação com o hospedeiro.

A abordagem comparativa utilizada demonstrou que as células NPTr responderam de forma específica a cada linhagem ou espécie de micoplasma e forneceu evidências de proteínas envolvidas na interação entre o *M. hyopneumoniae* patogênico com o hospedeiro e que podem ser determinantes da PES. Estudos proteômicos comparativos envolvendo as linhagens patogênicas e não patogênicas de *M. hyopneumoniae* e da bactéria comensal *M. flocculare* vêm fornecendo resultados promissores para o entendimento da patogênese de *M. hyopneumoniae* (Pinto et al., 2009b, Paes et al., 2017a, Paes et al., 2018), mas, pela primeira

vez, a resposta do hospedeiro em termos de secretoma, proteoma e proteínas secretadas por vesículas extracelulares foi caracterizada. Este trabalho forneceu evidências de uma extensiva lista de proteínas suínas e das linhagens de micoplasmas envolvidas na interação bactéria-hospedeiro e que podem ser exploradas quanto o seu papel na patogênese de *M. hyopneumoniae*.

M. hyopneumoniae e *M. flocculare* se aderem externamente às células epiteliais do trato respiratório suíno durante a infecção (Maes et al., 2017), portanto a linhagem celular NPTr, uma linhagem celular isolada de células da traqueia de um suíno (Ferrari et al., 2003), assemelha-se ao tipo celular ou ao ambiente em que as micoplasmas estão durante a infecção. Os resultados descritos aqui fornecem informações até então desconhecidas, visto que a maioria dos estudos envolvendo interação com *M. hyopneumoniae* e hospedeiro, utiliza células do sistema imune suíno. O uso de células epiteliais forneceu resultados de processos celulares locais em resposta à infecção, enquanto que estudos anteriormente publicados com células do sistema imune forneceram resultados sobre processos sistêmicos .

As proteínas secretadas pelas células do hospedeiro e pelas micoplasmas possuem um papel fundamental no desenvolvimento da PES, tendo em vista que a maioria das células de *M. hyopneumoniae* e *M. flocculare* não penetram nas células hospedeiras. No secretoma total, proteínas secretadas conhecidas como DAMPs (do inglês, *damage-associated molecule patterns*) foram identificadas exclusivamente na infecção com *M. hyopneumoniae* 7448 (cepa patogênica). DAMPs podem recrutar células do sistema imune ao local da infecção (Kono and Rock, 2008) e durante a infecção com *M. hyopneumoniae* ocorre a infiltração de neutrófilos e células mononucleares no tecido epitelial (Livingston et al., 1972, DeBey et al., 1992). Desse modo, estes resultados sugerem que as proteínas secretadas previamente à morte das células epiteliais podem ser um dos mecanismos que atuam recrutando estas células do sistema imune suíno ao local da infecção por *M. hyopneumoniae*.

Por outro lado, a adesão de *M. hyopneumoniae* às células suínas também pode desencadear processos intracelulares envolvidos na patogênese e no consequente desenvolvimento da PES. A comparação das proteínas intracelulares alteradas em resposta a uma linhagem patogênica de *M. hyopneumoniae* (7448) em comparação com as proteínas intracelulares alteradas durante a infecção com os micoplasmas não patogênicos (*M. hyopneumoniae* J ou *M. flocculare*) também permitiu a identificação de possíveis proteínas

e novos mecanismos envolvidos na patogênese de *M. hyopneumoniae*. Foi possível identificar proteínas alteradas exclusivamente durante a infecção com *M. hyopneumoniae* 7448 que possuem funções relacionadas com a homeostase de cálcio no retículo endoplasmático e mitocôndria. A desregulação da homeostase de cálcio já foi identificada como um mecanismo especifico da infecção com linhagens de *M. hyopneumoniae* patogênicos (Park et al., 2002) e os resultados obtidos fornecem novos alvos a serem estudados que podem estar envolvidos nesse mecanismo. Além da desregulação da homeostase de cálcio já descrita, outras proteínas alteradas especificamente a *M. hyopneumoniae* 7448 também revelaram um possível novo mecanismo de patogênese. A sobre-representação de proteínas envolvidas no estresse do retículo endoplasmático que podem levar à resposta terminal de proteínas malformadas (UPR, do inglês *unfolded proteins response*) evidenciou uma via de resposta potencialmente envolvida na morte celular observada durante a infecção com *M. hyopneumoniae* patogênico. No entanto, mais estudos são necessários para comprovar o papel de UPRs na morte celular de células infectadas com *M. hyopneumoniae*.

Proteínas suínas que desempenham funções intracelulares conhecidas e foram identificadas nas amostras de secretoma total e preditas como secretadas por vesículas, foram de fato identificadas posteriormente nas amostras de vesículas extracelulares. Desse modo, foi possível fornecer evidência experimental corroborativa da via de secreção por vesículas extracelulares de proteínas suínas identificadas no secretoma total. Esse resultado sugere que a presença de certas proteínas nos sobrenadante são de fato, produtos de secreção e não de contaminação com proteínas intracelulares, e permite futuras inferências do papel destas na interação *M. hyopneumoniae*-hospedeiro. Estas proteínas podem possuir funções *moonlight*, ou seja, podem desempenhar mais de uma função biológica ou biofísica, além da função já relacionada para a mesma (Mani et al., 2015). Infelizmente, proteínas possivelmente provenientes do soro fetal bovino e soro suíno utilizados nas culturas das células NPTr e na cultura de *M. hyopneumoniae*, respectivamente, também foram identificadas. A padronização de um protocolo diferente de lavagem das células suínas e de *M. hyopneumoniae* poderá evitar a contaminação das amostras com proteínas oriundas do soro bovino e suíno. Outra alternativa seria o uso do soro fetal bovino livre de vesículas, disponível comercialmente, e como consequência diminuição da complexidade da amostra,

eliminação da interferência de vesículas extracelulares do soro fetal bovino, melhorando os resultados de identificação do conteúdo proteico das vesículas extracelulares.

Além das proteínas suínas envolvidas na resposta aos diferentes micoplasmas identificadas e analisadas, também foram identificadas as proteínas das micoplasmas secretadas na presença ou não das células suínas. No secretoma total, foi possível observar que, após o contato com as células suínas, as micoplasmas secretam conjuntos diferentes de fatores de virulência, sendo que *M. hyopneumoniae* 7448 secreta mais adesinas e proteínas não-caracterizadas. Diferenças qualitativas e quantitativas nos repertórios de fatores de virulência secretados tinham sido demonstrados anteriormente (Paes et al., 2017a), porém os resultados descritos neste trabalho mostram uma relação entre as proteínas secretadas pelas micoplasmas, *M. hyopneumoniae* 7448, *M. hyopneumoniae* J e *M. flocculare*, e a resposta das células suínas contra as micoplasmas patogênicas e não patogênicas. Desse modo, o padrão de secreção diferencial das micoplasmas com diferença na patogenicidade pode ser um dos fatores determinantes na resposta diferencial das células suínas à infecção. Essa é a primeira vez que diferenças entre as proteínas secretadas pelas micoplasmas e diferenças na resposta das células suínas estão sendo correlacionadas.

O número de proteínas de *M. hyopneumoniae* identificadas nas vesículas extracelulares durante a interação com as células NPTr foi muito menor comparado com o observado no secretoma total. Os motivos possíveis podem ser: (i) vesículas extracelulares são apenas uma fração das proteínas totais secretadas pela micoplasma; (ii) o número mínimo de peptídeos para a validação de cada proteína foi maior nas amostras de vesículas extracelulares (4 peptídeos em vez de 1). Interessantemente, mais de 80% das proteínas de *M. hyopneumoniae* identificadas durante a interação com as células NPTr são fatores de virulência já descritos na literatura (Paes et al., 2018). Sob as mesmas condições, nenhum peptídeo ou proteína de *M. hyopneumoniae* foi identificado na cultura controle (sem células NPTr). Esses resultados sugerem que durante a interação com as células suínas, *M. hyopneumoniae* aumenta o número de vesículas extracelulares secretadas, e/ou, o conteúdo proteico com fatores de virulência das vesículas. Além disso, a secreção de fatores de virulência poderia contribuir para evasão do *M. hyopneumoniae* do sistema imune suíno, permitindo a infecção crônica pela bactéria.

6. PERSPECTIVAS

Proteínas envolvidas nas vias de resposta UPR e da regulação da homeostase de cálcio demonstraram abundância diferencial durante a infecção das células NPTr com *M. hyopneumoniae* 7448. Estas vias de sinalização são bem caracterizadas, porém nem todos os componentes destas vias mostraram abundância diferencial através na abordagem proteômica utilizada neste estudo. Deste modo, a perspectiva deste trabalho é estudar e validar a ativação destas vias de sinalização e sua importância na patogênese de *M. hyopneumoniae*. A identificação de diferença de abundância de todos os componentes das vias de sinalização por abordagem proteômica é limitada devido a complexidade da amostra, com a presença de todas as proteínas da célula suína, que impede a detecção por espectrometria de massas de componentes específicos. Portanto, duas alternativas podem ser aplicadas: (i) diminuir a complexidade da amostra proteica através de fracionamentos subcelulares das células NPTr infectadas com *M. hyopneumoniae* e análise destas frações por espectrometria de massas; (ii) detectar a diferença de expressão gênica, através de PCR quantitativo em tempo real, dos genes codificadores das proteínas componentes das vias de sinalização UPR e de regulação da homeostase de cálcio.

Além disso, as perspectivas incluem padronizar os ensaios de infecção e a preparação das amostras para melhorar a identificação das proteínas presentes nas vesículas extracelulares secretadas pelas células suínas e por *M. hyopneumoniae*. A identificação das proteínas presentes nas vesículas extracelulares mostrou contaminação com proteínas provenientes do soro fetal bovino e soro suíno utilizados na cultura das células NPTr e de *M. hyopneumoniae*. O uso de soro fetal bovino livre de vesículas extracelulares disponível comercialmente, ajuste no protocolo de lavagem das células NPTr e de *M. hyopneumoniae* após a cultura com meio suplementado com soro ou aumento do número de lavagens das vesículas extracelulares após a ultracentrifugação para remoção de proteínas contaminantes que não estão no interior das vesículas são alternativas para melhorar a identificação do conteúdo proteico das vesículas extracelulares. A visualização das vesículas extracelulares por microscopia eletrônica de transmissão também será realizada para confirmar a presença das vesículas extracelulares nas amostras após a ultracentrifugação.

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ANEXO

CURRICULUM VITAE RESUMIDO

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1. Dados pessoais

Nome: Fernanda Munhoz dos Anjos Leal Zimmer

Local e data de nascimento: Santa Maria, Rio Grande do Sul, Brasil, 02/07/1991

Endereço profissional: Universidade Federal do Rio Grande do Sul, Centro de Biotecnologia. Av. Bento Gonçalves, 9500 Prédio 43421 Sala 210.
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2. Formação acadêmica/Titulação

2015 - Atual Doutorado em andamento em Biologia Celular e Molecular (Conceito CAPES 7).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.
com período sanduíche em *Centers for Disease Control and Prevention* (Orientador: Hercules Moura).
Orientador: Henrique Bunselmeyer Ferreira.
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasil.

2013 - 2015 Mestrado em Biologia Celular e Molecular (Conceito CAPES 7).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Título: Análise de domínios diferenciais em proteínas de superfície ortólogas de *Mycoplasma hyopneumoniae* e *Mycoplasma flocculare*
Orientador: Henrique Bunselmeyer Ferreira.
Coorientador: Veridiana Gomes Virginio.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

2009 – 2012 Graduação em Biotecnologia.
Universidade Federal de Pelotas, UFPEL, Brasil.

3. Estágios

2010 – 2012 Vínculo: Bolsista, Enquadramento Funcional: Bolsista de Iniciação Científica, Carga horário: 20 h
Projeto: Desenvolvimento de teste diagnóstico para leptospirose suína através de ensaio imunoenzimático.
Universidade Federal de Pelotas, Centro de Biotecnologia, Laboratório de Imunodiagnóstico.

4. Prêmios e distinções

2018 - IOM2018 Student Travel Award, International Organization for Mycoplasmology.

2017 - ASM Student and Postdoctoral Travel Award, American Society of Microbiology.

2011 - Prêmio Jovem Pesquisador: 3º Lugar na Apresentação Oral na área de Ciência Agrárias, Universidade Federal de Pelotas.

5. Artigos completos publicados

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6. Resumos e trabalhos apresentados em congressos

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