MATHEUS MACHADO GUIDINI

CARACTERIZAÇÃO DE VESÍCULAS EXTRACELULARES PRODUZIDAS POR *Actinobacillus pleuropneumoniae* EM RESPOSTA A DIFERENTES CONDIÇÕES DE ESTRESSE

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

Orientador: Denise Mara Soares Bazzolli

Coorientador: Hilário Cuquetto Mantovani

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Denise Mara Soares Bazzolli
Orientador

Aos meus avós, pais e irmãos.

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*"*Ninguém ignora tudo. Ninguém sabe tudo. Todos nós sabemos alguma coisa. Todos nós ignoramos alguma coisa. Por isso aprendemos sempre*"*.

(Paulo Freire)

RESUMO

GUIDINI, MATHEUS MACHADO, M.Sc., Universidade Federal de Viçosa, março de 2023. **CARACTERIZAÇÃO DE VESÍCULAS EXTRACELULARES PRODUZIDAS POR *Actinobacillus pleuropneumoniae* EM RESPOSTA A DIFERENTES CONDIÇÕES DE ESTRESSE**. Orientadora: Denise Mara Soares Bazzolli. Coorientador: Hilário Cuquetto Mantovani.

A família *Pasteurellaceae* engloba diversas espécies de bactérias Gram-negativas, algumas das quais são patogênicas para humanos e animais de produção. Membros dessa família são responsáveis por doenças que causam impacto econômico significativo na indústria suinícola, resultando em desafios consideráveis e altos custos associados ao uso de antimicrobianos. Essas bactérias frequentemente manifestam resistência a múltiplas drogas, o que dificulta ainda mais o tratamento. Entre as principais enfermidades na cadeia suinícola, a pleuropneumonia suína (PPS) destaca-se como uma das mais comuns. *Actinobacillus pleuropneumoniae* (App) é uma bactéria Gram-negativa, anaeróbia facultativa, pertencente à família Pasteurellaceae, sendo o agente etiológico da pleuropneumonia suína. Esta bactéria possui diversos fatores de virulência, como cápsula, lipopolissacarídeo (LPS), produção de sideróforos para captação de ferro e toxinas Apx. Além disso, é capaz de produzir vesículas extracelulares (VEs). Estudos anteriores destacaram a importância das VEs como um fator de virulência para App, mas ainda faltam informações sobre as condições que induzem a produção de VEs, bem como a transferência de material genético carregado por essas vesículas. Portanto, os objetivos deste trabalho foram investigar a vesiculação por *A. pleuropneumoniae* MV780, sorotipo 8, sob diferentes condições de estresse, e determinar se as VEs produzidas transportam material genético, um novo mecanismo de transferência horizontal de genes para App. As VEs de App foram obtidas em diferentes fases do crescimento bacteriano e em diferentes condições de estresse: por privação de ferro e na presença de antimicrobianos no meio de cultivo. Posteriormente, foram realizados testes de vesidução para verificar a capacidade de transferência de material genético por VEs de App. As vesículas foram investigadas quanto ao tamanho e dispersão, morfologia, conteúdo proteico e quantificação. Por fim, realizamos análises moleculares para detecção de marcadores moleculares que indicam a presença do plasmídeo p780 nas VEs produzidas e o possível potencial de transferência para outros isolados de App por vesidução. Nossos resultados indicaram que as vesículas produzidas por App não apresentam diferenças significativas quanto à morfologia; porém, tamanho e dispersão variaram nas diferentes fases de crescimento e em

diferentes condições de estresse. Nossos dados indicam que tanto a fase de crescimento quanto as condições de estresse influenciam diversos aspectos da produção de VEs, incluindo quantidade e perfil proteico. Evidenciamos que App produz mais VEs em condições de estresse, independentemente das fases de crescimento. As VEs produzidas apresentam material genético intracelular protegido da ação de nucleases, e o plasmídeo p780 é empacotado por VEs nas diferentes condições estudadas. Demonstramos o potencial de transferência deste plasmídeo para outro isolado de App, comprovando assim que App possui a capacidade de transferência de material genético carreado por vesículas extracelulares. Este trabalho é pioneiro na produção e caracterização de VEs em condições de privação de ferro e na presença de agentes antimicrobianos no meio de cultivo. Além disso, demonstramos pela primeira vez para a espécie a transferência de material genético por vesidução em App.

Palavras-chave: *Pasteurellaceae. Actinobacillus pleuropneumoniae.* Vesículas Extracelulares. Vesiculação. Vesidução.

ABSTRACT

GUIDINI, Matheus Machado, M.Sc., Universidade Federal de Viçosa, March, 2024. **CHARACTERIZATION OF EXTRACELLULAR VESICLES PRODUCED BY *Actinobacillus pleuropneumoniae* IN RESPONSE TO DIFFERENT STRESS CONDITIONS**. Adviser: Denise Mara Soares Bazzolli. Co-adviser: Hilário Cuquetto Mantovani.

The *Pasteurellaceae* family encompasses various Gram-negative bacteria, some of which are pathogenic to humans and production animals. Members of this family are responsible for diseases that have a significant economic impact on the swine industry, leading to considerable challenges and high costs associated with the use of antimicrobials. These bacteria often exhibit resistance to multiple drugs, further complicating treatment. Among the primary diseases in the swine industry, swine pleuropneumonia (SPP) stands out as one of the most common. *Actinobacillus pleuropneumoniae* (App) is a Gram-negative, facultative anaerobic bacterium belonging to the *Pasteurellaceae* family, serving as the etiological agent of swine pleuropneumonia. This bacterium possesses various virulence factors, such as a capsule, lipopolysaccharide (LPS), siderophore production for iron uptake, and Apx toxins. Additionally, it is capable of producing extracellular vesicles (EVs). Previous studies have emphasized the importance of EVs as a virulence factor for App, but information is still lacking regarding the conditions that induce EV production and the transfer of genetic material carried by these vesicles. Therefore, the objectives of this study were to investigate vesiculation by *A. pleuropneumoniae* MV780, serotype 8, under different stress conditions and determine if the produced EVs transport genetic material, representing a novel mechanism for horizontal gene transfer in App. EVs from App were obtained at different bacterial growth phases and under various stress conditions, including iron deprivation and the presence of antimicrobials in the culture medium. Subsequently, vesiculation tests were conducted to verify the capacity for genetic material transfer by App EVs. The vesicles were examined for size and dispersion, morphology, protein content, and quantification. Finally, molecular analyses were performed to detect molecular markers indicating the presence of the p780 plasmid in the produced EVs and the potential for transfer to other App isolates through vesiculation. Our results indicated that vesicles produced by App showed no significant differences in morphology, but size and dispersion varied in different growth phases and under different stress conditions. Our data suggest that both the growth phase and stress conditions influence various aspects of EV

production, including quantity and protein profile. We demonstrated that App produces more EVs under stress conditions, regardless of growth phases. The produced EVs contain intracellular genetic material protected from nuclease action, and the p780 plasmid is packaged by EVs under the studied conditions. We showcased the potential transfer of this plasmid to another App isolate, confirming that App has the capability to transfer genetic material via extracellular vesicles. This study is pioneering in the production and characterization of EVs under iron deprivation and the presence of antimicrobial agents in the culture medium. Additionally, we demonstrated, for the first time in the species, the transfer of genetic material through vesiculation in App.

Keywords: *Pasteurellaceae*. *Actinobacillus pleuropneumoniae*. Extracellular Vesicles. Vesiculation. Vesiduction.

SUMÁRIO

INTRODUÇÃO GERAL 13
Suinocultura: Aspectos Gerais e Desafios 13
Actinobacillus pleuropneumoniae: patogenicidade e virulência 14
Vesículas extracelulares: um mundo de possibilidades 17
Vesículas extracelulares e transferência horizontal de genes 20
REFERÊNCIAS BIBLIOGRÁFICAS 23
OBJETIVO GERAL 35
OBJETIVOS ESPECÍFICOS 35
MANUSCRIPT 36
Vesiculation by *Actinobacillus pleuropneumoniae* is stimulated by different stress conditions and mobilizes antibiotic resistance genes by vesiduction 36
Introduction 37
Material and Methods 40
Bacteria strains and growth conditions 40
Determination of growth curve in stress conditions 41
Extracellular vesicles extraction and purification 41
EVs characterization 42
EVs Morphology by Transmission Electron Microscopy (TEM) 42
EVs size by Dynamic Light Scattering (DLS) 42
EVs quantification 42
Detection of p780 plasmid in EVs produced by *A. pleuropneumoniae* 43
Vesiduction assay 44
Confirmation of Transvesiductants by PCR 45
Statistical Analysis 45
Results 45
Phenotypic Growth Analysis 45
Morphology of EVs *A. pleuropneumoniae* 47
Protein profile of EVs produced by *A. pleuropneumoniae* 51
Production of EVs 52
Detection of the molecular marker p780 in *A. pleuropneumoniae* EVs. 55
Vesiduction Acting on Horizontal Gene Transfer (HGT) 57
Discussion 58
Acknowledgments 60
Funding information 61

Conflicts of interest 61
Supplementary material 62
References 62
Conclusão 66

INTRODUÇÃO GERAL

Suinocultura: Aspectos Gerais e Desafios

A carne suína é uma das fontes de proteína animal mais consumidas no Brasil e no mundo (VICENTE et al., 2021). A suinocultura é um setor de destaque nacional nas atividades econômicas do setor alimentício do país (ABPA, 2022). Nos últimos anos, o cenário global da produção de carne suína foi impactado pela propagação da Peste Suína Africana (PSA) em vários países produtores dessa commodity (MASON-D'CROZ et al., 2020). Além disso, esse mercado enfrentou desafios decorrentes da pandemia por COVID-19 nos anos de 2019, 2020 e 2021 (USDA, 2022). Apesar do aumento nas exportações, o Brasil mantém sua posição como o quarto maior produtor e exportador global de carne suína (ABPA, 2022). No âmbito nacional, Minas Gerais é o quarto maior produtor de carne suína, sendo responsável por 11,16% de toda a produção nacional (ABPA, 2022). Em 2022, conforme relatado no balanço geral da Associação Brasileira de Proteína Animal, foram produzidas 5 milhões de toneladas de carne suína em todo o território nacional. Desse montante, 3,9 milhões de toneladas foram destinadas ao consumo interno, enquanto 1,1 milhão de toneladas foram destinadas à exportação (ABPA, 2022).

A demanda mundial por carne suína apresenta um crescente aumento, e espera-se que na próxima década seja impulsionada pelo aumento de renda e população em diversas regiões do mundo, principalmente na Ásia (YU et al., 2022). O crescente aumento na cadeia produtiva de carne suína é seguido por um expressivo aumento no lucro de empresas exportadoras de carnes e insumos em todo o mundo (BOKUSHEVA & KIMURA, 2019). Em contraste com toda a expectativa de maior crescimento, a cadeia de produção suinícola ainda apresenta os mesmos desafios, pois utiliza métodos de produção intensiva e em larga escala, o que normalmente onera a produção devido à necessidade de aumento de investimentos para assegurar o manejo sanitário desses animais (DELSART et al., 2020). Animais de produção, como os suínos, são hospedeiros ou reservatórios para muitas zoonoses já documentadas (BAUDON et al., 2015). Na suinocultura, as doenças respiratórias representam um dos desafios associados à criação intensiva, a qual facilita a rápida transmissão de doenças infecto-respiratórias entre animais confinados em pequenos espaços físicos (FOURNIÉ et al., 2015).

Diversos agentes etiológicos têm a capacidade de provocar doenças respiratórias nas diferentes fases da produção, acarretando problemas no rebanho e resultando em perdas

econômicas significativas (RAMPELOTTO et al., 2022). A maioria dos agentes virais que possuem a capacidade de causar doenças respiratórias em suínos ocasiona uma pneumonia intersticial típica (HAIMI-HAKALA et al., 2017). Dentre os agentes virais, destacam-se o vírus da síndrome reprodutiva e respiratória suína (PRRSV), o vírus da influenza suína (SIV), o coronavírus respiratório (PRCV) e o circovírus suíno tipo 2 (PCV-2) (HAIMI-HAKALA et al., 2017; RAMPELOTTO et al., 2022). Em outra perspectiva, inúmeros agentes etiológicos estão relacionados a doenças infecto-respiratórias bacterianas em suínos, sendo eles: *Actinobacillus pleuropneumoniae, Pasteurella multocida, Mycoplasma hyopneumoniae, Streptococcus suis, Bordetella bronchiseptica* e *Haemophilus parasuis* (BROMBILLA et al., 2019).

Dentre as principais doenças reportadas na cadeia suinícola, a pleuropneumonia suína (PPS) é uma das enfermidades mais frequentemente relatadas nas granjas (PEREZCHICA et al., 2023). A PPS possui uma distribuição global, sendo relatada em todos os países que apresentam métodos de produção em larga escala, o que evidencia a inexistência de um controle efetivo da doença (STRINGER et al., 2022).

A pleuropneumonia suína é identificada por apresentar broncopneumonia necrosante e hemorrágica, podendo apresentar uma pleurite fibrinosa associada (PEREIRA et al., 2018). O agente etiológico da PPS é a bactéria *Actinobacillus pleuropneumoniae* (App). Essa bactéria possui diferentes modos de transmissão, incluindo a propagação por aerossóis em curtas distâncias, contato direto e indireto (LOERA-MURO et al., 2013; SASSU et al., 2017).

Actinobacillus pleuropneumoniae: patogenicidade e virulência

App é uma bactéria Gram-negativa, cocobacilar, anaeróbia facultativa, formadora de cápsula, pertencente ao filo Pseudomonadota, classe Gammaproteobacteria, ordem Pasteurellales, família *Pasteurellaceae*. Sua forma pode variar de cocoide a bacilar, com tamanho usual de 0,4 x 1,0 μm; não formam esporos e não apresentam motilidade (DONACHIE; LAINSON; HODGSON, 1995). App pode ser dividida em dois biótipos diferentes com base em suas exigências de nicotinamida adenina dinucleotídeo (NAD): o biótipo 1 requer NAD externo, enquanto o biótipo 2 tem a capacidade de sintetizar NAD na presença de nucleotídeos específicos de piridina ou de seus precursores (BOSSÉ et al., 2002). Atualmente, são descritos 19 sorotipos desta bactéria, com diferenças em relação à organização dos genes envolvidos na produção de cápsula e diferenças relacionadas às propriedades antigênicas dos polissacarídeos da cápsula (STRINGER et al., 2021). App é o agente causador da pleuropneumonia suína. No entanto, em muitos animais saudáveis, ela pode ser encontrada

como membro da microbiota normal de suínos, normalmente encontrada nas tonsilas e no trato respiratório superior de animais pertencentes ao gênero *Sus*, sendo relatado a presença desta bactéria na cavidade nasal, tonsilas, cavidade auditiva média e pulmões de suínos infectados (ZHAO et al., 2008; SASSU et al., 2017). A PPS pode ser desencadeada por todos os sorotipos de App já identificados. Entretanto, a gravidade da doença está associada à virulência que é peculiar mediante o panorama de toxinas e outros fatores produzidos por cada sorotipo (FREY et al., 2011).

A ocorrência de biótipos e sorotipos diferentes de App mostra notáveis variações conforme as diferentes regiões globais (SÁRKÖZI et al., 2018). Na Europa, as cepas do sorotipo 2 predominam em geral em áreas específicas, como na Espanha, os sorotipos 2, 4 e 7 são os mais frequentemente relatados (MALDONADO et al., 2009). Na Suíça, o sorotipo 2 também é o mais frequente relatado (STÄRK et al. em 2007). Dentre esses sorotipos, o sorotipo 2, 3, 6–8, 10, 12 e 9 são predominantes no Reino Unido (O'NEILL et al., 2010). No Brasil, os sorotipos de maior prevalência são o 5, 7 e o 8, sendo o sorotipo 8 o mais relatado no Estado de Minas Gerais (ROSSI et al., 2013; KUCHIISHI et al., 2023).

A aquisição de ferro é crucial para o crescimento, metabolismo e colonização bacteriana, sendo essencial para a sobrevivência da maioria das bactérias (COOK-LIBIN et al., 2022). App utiliza diferentes estratégias específicas para captar ferro no ambiente celular, podendo utilizar a transferrina suína, hemoglobina e sideróforos fornecidos exogenamente como fontes exclusivas de ferro intracelular (NIVEN et al., 1989; BÉLANGER et al., 1995; DIARRA et al., 1996; BALTES et al., 2002).

Em uma das estratégias utilizadas por *A. pleuropneumoniae* ocorre a expressão de duas proteínas distintas de ligação à transferrina suína conhecidas como TbpA/TbpB (de 110 kDa e 60 kDa, respectivamente), envolvidas na captação de transferrina em condições de restrição de ferro (MIKAEL et al., 2003). Ambas as proteínas são específicas para a transferrina suína, o que evidencia a relação evolutiva de App para com seu único hospedeiro mamífero (MIKAEL et al., 2002). TbpA e TbpB são proteínas coexpressas com o complexo de proteínas de transporte da membrana interna ExbBD. Este complexo, juntamente com TonB, desempenha um papel crucial no transporte de ferro através da membrana interna celular (TONPITAK et al., 2000). Já foi descrito um transportador AfuABC, que é capaz de transportar ferro através da membrana citoplasmática de App (CHIN et al., 1996). Archambault em 2003, descreveu pela primeira vez duas proteínas com tamanhos 75 e 104 kDa, capazes de se ligarem a hemina

e hemoglobina, que são reguladas pela presença do ferro. App possui também possui um sistema de aquisição de ferro por meio do operon *fhuCDBA*, que codifica proteínas de membrana externa e interna FhuA (78,9 kDa), FhuD (35,6 kDa), FhuC (28,5 kDa) e Fhu (69,4 kDa). FhuA está associada a outras três proteínas, FhuD (35,6 kDa), FhuC (28,5 kDa) e Fhu (69,4 kDa), todas relacionadas à ligação da transferrina e à translocação e transporte de hidroxamato férrico. A lipoproteína FhuA, pertencente à família dos hidroxamatos, atua como um sideróforo na interação com App (MIKAEL et al. 2002, 2003; SRIKUMAR et al., 2004). A importância da captação e metabolismo de ferro em *Actinobacillus pleuropneumoniae* é evidenciada pelo envolvimento de mais de 50 genes nessas funções, conforme descrito por Chen e colaboradores (2022), o que mostra a importância deste micronutriente para a colonização e sobrevivência de App.

Estudos envolvendo espécies da família *Pasteurellaceae* descreveram a presença de diversos plasmídeos relacionados à resistência a antimicrobianos (SILVA et al., 2022). Já é relatado que a resistência antimicrobiana à tetraciclina é amplamente disseminada entre isolados de A. pleuropneumoniae em todo o mundo (BLANCO et al., 2006; ARCHAMBAULT et al., 2012). Apesar disso, as tetraciclinas, incluindo a tetraciclina, doxiciclina, oxitetraciclina e outros derivados, ainda são constantemente usadas no tratamento contra a pleuropneumonia suína. Essa abordagem é mais eficaz para atenuar a gravidade dos sintomas clínicos, reduzindo a taxa de mortalidade e a propagação da infecção (DOREY et al., 2017; VAILLANCOURT et al., 2021). O uso desses antibióticos na prática veterinária ainda é sustentado pelo fato de que esses medicamentos possuem amplo espectro para tratamento contra bactérias Gram-negativas e Gram-positivas, possuindo assim uma extensa aplicação na medicina veterinária, sendo utilizados tanto para fins profiláticos quanto para a promoção do crescimento em animais de produção (CHOPRA & ROBERTS, 2001).

Diversos fatores de virulência foram identificados em *Actinobacillus pleuropneumoniae*, abrangendo adesão, aquisição de nutrientes, indução de lesões pulmonares, evasão do sistema imunológico e persistência (SASSU et al., 2017). Os diferentes sorotipos de App podem exibir padrões diferentes de virulência, sendo que a variação pode ser atribuída tanto à ausência de genes relacionados à virulência quanto à expressão diferencial de exotoxinas secretadas por App (STRINGER et al., 2021). Polissacarídeos capsulares (cápsula), proteínas de captação de ferro (sideróforos), lipopolissacarídeos (LPS), toxinas (Apx), plasmídeos e produção de vesículas extracelulares (do inglês *Extracellular vesicles* - EVs) são considerados

os principais fatores relacionados à virulência em *A. pleuropneumoniae* (ABASCAL et al., 2000; BOSSÉ et al., 2002; CHIERS et al., 2010; FREY, 2011; NAHAR et al., 2021 ZHU et al., 2022).

Vesículas extracelulares: um mundo de possibilidades

Adaptação e a sobrevivência bacteriana em diferentes ambientes demandam uma robusta capacidade de se ajustar e usar estratégias diversas (GILL et al., 2018). Neste panorama, a produção de vesículas extracelulares (VEs) pelos microrganismos representam uma forma eficaz de comunicação, nutrição e proteção aos diferentes desafios do ambiente (BRAUD et al., 2020).

No contexto bacteriano, as VEs são nanoestruturas globulares derivadas da superfície celular e podem ser produzidas por diferentes grupos de bactérias, incluindo bactérias Gram-positivas, bactérias Gram-negativas, micoplasmas e bacilos ácido-álcool resistentes. (SALJE, J. et al., 2014; BROWN et al., 2015; GILL et al., 2018). A produção de vesículas é contínua e são produzidas ao longo de todos os estágios do crescimento bacteriano, em condições ótimas de crescimento e em resposta a diferentes tipos de estresse, constituindo um processo celular ubíquo nos e três domínios da vida (DEPLETEAU & BRIEGEL, 2019).

VEs liberadas por bactérias Gram-negativas foram inicialmente denominadas vesículas de membrana externa (do inglês *Outer Membrane Vesicles*) e foram relatadas pela primeira vez na década de 1960, através de estudos de microscopia eletrônica, isoladas a partir de sobrenadante livre de células de Escherichia coli, sendo consideradas artefatos celulares (ROTHFIELD, 1965). Atualmente, é consenso que as bactérias podem produzir diferentes tipos de vesículas extracelulares, uma vez que não está mais restrito às bactérias Gram-negativas, o que vai além da caracterização de OMVs (JAN, 2017; TOYOFUKU et al., 2019; WEN et al., 2023; TOYOFUKU et al., 2023). Especificamente no caso das bactérias Gram-negativas, as VEs são produzidas durante todas as fases de crescimento, com tamanhos que podem variar entre 10 e 300 nm (JAN, 2017). Já é descrito para muitas bactérias Gram-negativas que condições de estresse e quorum sensing influenciam na produção de VEs (ABRAMOWICZ et al., 2019; MUNHOZ et al., 2020). As VEs de Gram-negativas liberadas carregam diferentes biomoléculas como proteínas, moléculas de sinalização, lipopolissacarídeos (LPS), proteínas citoplasmáticas e periplasmáticas, ácidos nucleicos (DNA e RNA) e, frequentemente, outros fatores relacionados à patogênese e virulência (VELLA, B. D.; SCHERTZER, J., 2014; ANAND, D.; CHAUDHURI, A., 2016; DOWLING et al., 2016).

Hoje, mesmo com todas as tecnologias de estudo das VEs ainda existem lacunas e divergências sobre a biogênese, vias de liberação, tamanho médio, carga e função (SABATKE et al., 2023). No contexto atual as VEs podem ser descritas em diferentes categorias de acordo com a composição: vesículas de membrana do tipo B, que correspondem às vesículas de membrana externa (OMVs); as de membrana externa-interna (OIMVs); e as de membrana citoplasmática (CMVs) (TOYOFUKU et al., 2023). As vesículas do tipo B estão associadas principalmente aos distúrbios no envelope celular (TOYOFUKU et al., 2018). Já as vesículas de membrana do tipo E são classificadas em vesículas de membrana citoplasmática via lise (ECMVs), de membrana externa explosivas (EOMVs) e, por fim, vesículas explosivas de membrana externa-interna (EOIMVs) (TOYOFUKU et al., 2019; LIU et al., 2022; TOYOFUKU et al., 2023).

Existem dois métodos para a formação das VEs: a formação das VEs líticas (ocorre durante a lise celular) e as VEs ditas não-líticas (através da formação de bolhas na membrana externa) (TOYOFUKU et al., 2023). A biogênese das VEs ainda é tema de debate, pois não há um modelo específico que descreva o mecanismo de veiculação. Quatro mecanismos que podem estar possivelmente envolvidos com a produção de VEs em bactérias foram descritos. Estes são a redução nas conexões entre a membrana externa e o peptidoglicano, através da quebra de ligações peptídicas existentes, distúrbio na curvatura da membrana externa, aumento na pressão periplasmática por meio de enriquecimento de proteínas mal dobradas e, por fim, através da rotação flagelar (MCBROOM et al., 2006; WESSEL et al., 2013; ASCHTGEN et al., 2015; ROIER et al., 2016; JUODEIKIS & CARDING, 2022). A produção de vesículas em bactérias Gram-negativas em geral corresponde a uma redução da estabilidade do arcabouço da membrana celular. Isso ocorre como resultado das ligações entre a membrana externa, o peptidoglicano e a membrana interna, resultando na desestabilização do citoesqueleto bacteriano. Esse processo leva à formação de projeções na membrana externa, seguida pela expansão e subsequente excisão da camada de fosfolipídios, resultando na liberação das VEs (TOYOFUKU et al., 2023).

As VEs exercem uma variedade de funções fisiológicas e celulares, incluindo comunicação intra e interespécies, transferência horizontal de genes, formação de biofilme, resistência a antibióticos, transporte de toxinas e fatores de virulência, modulação da resposta imune atuando como fatores imunomoduladores em seus hospedeiros e redução de estresse

celular. (ACEVEDO et al., 2014; KLIMENTOVÁ & STULÍK, 2014; GILL et al., 2019; WANG et al., 2020; ZHU et al., 2022).

O aumento na produção de vesículas está associado às diferentes condições de crescimento: como a presença de antimicrobianos, depleção de nutrientes como presença de agentes quelantes de ferro, alteração de pH e temperatura, entre outros (ORENCH-RIVERA & KHUEN, 2016; ROIER et al., 2016; BROWN et al., 2021; COMBO et al., 2022). Fatores ambientais, incluindo aqueles que os patógenos encontram durante a fase inicial da infecção, como a baixa concentração de ferro, frequentemente exercem impacto na produção e composição das vesículas liberadas por bactérias Gram-negativas (KUEHN, M.; KESTY, N. et al., 2005). Devido à sua baixa solubilidade em água na presença de oxigênio e pH neutro, o ferro férrico não é encontrado de forma livre no hospedeiro (WEINBERG., 1999).

A importância do ferro para bactérias já é conhecida, uma vez que desempenha um papel crucial como micronutriente essencial para a sobrevivência de microrganismos. (GERNER et al., 2020). Assim, durante as fases iniciais de uma infecção, o hospedeiro restringe o acesso ao ferro, e as respostas imunes inatas trabalham reduzindo ainda mais a disponibilidade desse elemento, inibindo o desenvolvimento de potenciais patógenos (GANZ., 2009; NAIRZ et al., 2020). Neste contexto, devido à limitação de ferro enfrentada por muitos patógenos microbianos ao entrarem em contato com as membranas de mucosas e tecidos do hospedeiro, já foi relatado que os patógenos podem aumentar a produção de VEs nas fases iniciais da infecção garantindo o sucesso de sua sobrevivência *in vivo*, em baixas concentrações de ferro disponíveis no ambiente celular do hospedeiro (MCDONALD et al., 2013; ROSALES et al., 2014; HONG et al., 2019).

Em diversos países, os agentes antimicrobianos mais comumente utilizados na prática clínica para tratar doenças respiratórias abrangem as tetraciclinas, macrolídeos/lincosamidas, aminoglicosídeos, beta-lactâmicos e trimetoprima/sulfonamidas (ECONOMOU & GOUSIA, 2015). Neste panorama, os lipídios de membrana e proteínas que estão envolvidos na síntese ou processamento de componentes da membrana são alvos atraentes para antibióticos (YANG et al., 2023). Diversos estudos já elucidaram que as VEs podem atuar como veículos de proteção para as bactérias, atuando contra os efeitos de antibióticos (SCHWECHHEIMER & KUEHN, 2015; KIM et al., 2018). Esse tipo de mecanismo pode ocorrer por meio de três fatores: (I) transferência horizontal de genes (THG) de resistência, (II) inativação de antimicrobianos por degradação enzimática e (III) pela degradação/sequestro direto dos antibióticos (CIOFU, 2000;

SCHAAR et al., 2011; FULSUNDAR et al., 2014; KULKARNI et al., 2015). O mecanismo exato pelo qual as VEs atuam contra antibióticos ainda não foi completamente caracterizado; no entanto, diversas pesquisas prévias já destacaram que a exposição a determinados estressores fisiológicos ou ambientais, como o tratamento com antibióticos, afeta a secreção de vesículas pelas células bacterianas (MACDONALD et al., 2013; BAUWENS et al., 2017). MACDONALD (2013) relatou o aumento do nível da secreção de VEs por *Pseudomonas aeruginosa* quando tratada com polimixina B. Manning e Kuehn (2011) descreveram que a exposição à colistina resultou em uma maior formação de VEs em uma linhagem de *E. coli*.

Em App, já existem registros sobre a produção de VEs, sendo os primeiros datados das décadas de 80 e 90, os pioneiros foram os estudos de Jacques et al. (1988) e Rosendal e MacInnes (1990), nos quais foram feitos os primeiros relatos da produção de VEs por App. Negrete e colaboradores (2000) identificaram toxinas Apx1 e II sendo carreadas por VEs em App. Já existem estudos voltados à atuação de VEs liberadas por *A.pleuropneumoniae* com o objetivo de entender o seu padrão antigénico para o desenvolvimento de plataformas vacinais (ANTENUCCI et al., 2019). Recentemente, Silva et al. (2022; 2023) relataram novas informações sobre a produção de VEs e com destaque na presença de RNAs pequenos reguladores nas VEs, inclusive com identificação de novos sRNAs em App. No entanto, a produção de VEs de forma natural frente a condições de estresse ainda não foi documentado, informações estas são muito importantes porque a partir do conhecimento mais aprofundado sobre produção, composição e carga de VEs por App será possível de fato a construção de plataformas vacinais para serem aplicadas no controle de doenças respiratórias em suínos.

Vesículas extracelulares e transferência horizontal de genes

A Transferência Horizontal de Genes (THG), também conhecida como Transferência Lateral de Genes (TLG), refere-se à transferência da informação genética entre organismos que não estão necessariamente relacionados (BURMEISTER, 2015). Este mecanismo ultrapassa a limitação natural da reprodução e herança genética tradicional entre progenitores e descendentes, que é denominada Transferência Vertical de Genes (TVG) (KEELING & PALMER, 2008). A THG é considerada um pilar fundamental para a evolução molecular bacteriana, trabalhando em conjunto com a mutação (ARNOLD et al., 2021). Historicamente, três principais mecanismos de THG foram propostos e consolidados: transformação natural, conjugação e transdução (SOLER & FORTERRE, 2020). Esses processos desempenham

papéis essenciais na transmissão de informações genéticas entre diferentes organismos, contribuindo para a plasticidade genômica e diversidade genética (SILVA et al., 2022). A transformação natural foi relatada pela primeira vez em 1928, com o experimento de Griffith com *Streptococcus pneumoniae* (GRIFFITH, 1928). Esse mecanismo envolve a absorção livre de DNA do ambiente por células bacterianas competentes (STEWART & CARLSON, 1986).

A conjugação bacteriana, descoberta por Lederberg e Tatum, é um mecanismo que envolve a troca unidirecional de DNA por contato direto entre uma célula doadora e uma célula receptora (LEDERBERG & TATUM, 1946). A transferência de material genético durante o processo de conjugação pode ser mediada por dois tipos de elementos genéticos: os plasmídeos conjugativos, que se replicam autonomamente no citoplasma, e os elementos integrativos e conjugativos, também chamados de transposons conjugativos, os quais costumam permanecer integrados no genoma do hospedeiro (GROHMANN et al., 2003; FERNANDO et al., 2010; VIROLLE et al., 2020).

Por fim, a transferência de DNA entre células utilizando bacteriófagos como vetores representa um terceiro mecanismo bem estabelecido, conhecido como transdução (LANG et al., 2007; DAUBIN et al., 2016). Esse processo foi inicialmente demonstrado por Zinder e Lederberg em 1952 (ZINDER, N. D.; LEDERBERG, J., 1952). Nesse mecanismo, ao final do ciclo de replicação viral, ocorre a lise da célula hospedeira bacteriana, resultando no empacotamento de pequenos fragmentos do DNA do genoma hospedeiro nas partículas virais infecciosas. Essas partículas, por sua vez, infectam células bacterianas próximas, e o DNA pode então ser injetado em outro indivíduo, no lugar do DNA do vírus (LANG et al., 2012).

Mais recente um quarto mecanismo de THG foi proposto e aceito sendo denominado Vesidução, o qual envolve a transferência de DNA via VEs para uma célula receptora (SOLER, N.; FORTERRE, P, 2020). Neste contexto, o primeiro relato da atuação das VEs no transporte de material genético remonta aos anos 80, quando o transporte de DNA por meio de VEs foi observado em *Haemophilus influenzae* e *Neisseria gonorrhoeae* (BARANY et al., 1983; DORWARD et al., 1989).

Estudos realizados por Kahn (1983) e Dorward (1989), respectivamente, demonstraram que as VEs protegem o DNA de endonucleases de restrição, evidenciando assim a importância das VEs como mecanismo de transporte de DNA de célula para célula. Yaron (2000) destacou a transferência de genes de resistência de *Escherichia coli* carreados por VEs para outras espécies de bactérias enteropatogênicas. Hua (2022) sugeriu a possibilidade de

transmissão de genes de virulência por meio de vesículas de membrana externa em *Klebsiella pneumoniae*, resultando na formação de linhagens hipervirulentas que também apresentam resistência a importantes antimicrobianos.

A relevância histórica e biológica da vesidução na evolução adaptativa microbiana ainda não foi desvendada (SHI et al., 2021). Apesar da falta de uma compreensão abrangente de seu papel específico na evolução, a presença generalizada de VEs sugere que esse fenômeno pode desempenhar uma função importante na dinâmica microbiana e na adaptação a diferentes ambientes (SOLER, N.; FORTERRE, P., 2020). A vesidução, como um novo mecanismo de transferência horizontal de genes, pode contribuir significativamente para a compreensão inicial da transferência genética por meio de VEs, delineando assim a importância desse processo na comunicação genética entre as células bacterianas.

Estudos envolvendo espécies da família *Pasteurellaceae* descreveram a presença de diversos plasmídeos relacionados à resistência a antimicrobianos na família (SILVA et al., 2022). Já é relatado que a resistência antimicrobiana à tetraciclina é amplamente disseminada entre isolados de *A. pleuropneumoniae* em todo o mundo (BLANCO et al., 2006; ARCHAMBAULT et al., 2012). Apesar disso, as tetraciclinas, incluindo a tetraciclina, doxiciclina, oxitetraciclina e outros derivados, ainda são constantemente usadas no tratamento contra a pleuropneumonia suína, abordagem essa mais eficaz para atenuar a gravidade dos sintomas clínicos, reduzindo a taxa de mortalidade e propagação da infecção (DOREY et al., 2017; VAILLANCOURT et al., 2021).

No contexto da pesquisa desenvolvida por nosso grupo, Silva (2015) identificou e caracterizou dois plasmídeos em linhagens de App sorotipo 8 *MV518* e *MV780*, denominados p518 e p780, respectivamente. Esses plasmídeos conferem resistência aos antimicrobianos florfenicol e tetraciclina, respectivamente. Estudos subsequentes indicaram que o plasmídeo p780 não é mobilizável e não possui o aparato completo de genes necessários para torná-lo conjugativo. Além disso, foi observada uma similaridade de 100% com outro plasmídeo isolado em *Pasteurella multocida*, na Espanha, sugerindo uma disseminação do p780 entre diferentes espécies da família *Pasteurellaceae* (LI et al., 2018). Esse cenário ressalta a importância da compreensão dos mecanismos de resistência e transferência gênica na eficácia da resistência antimicrobiana mediada por VEs bacterianas, uma vez que como não se trata de plasmídeos mobilizáveis algum outro mecanismo está envolvido na transferência gênica. Nenhum trabalho ainda realizou uma descrição e caracterização completa da composição das VEs produzidas por

App em condições de estresse, condições essas normalmente encontradas na interação patógeno-hospedeiro. Desta forma, este trabalho prevê a caracterização da produção de VEs em *A. pleuropneumoniae* em resposta a diferentes condições de estresse. Assim, com base nas informações supracitadas anteriormente, nossa hipótese é que diferentes condições de estresse estimulam a vesiculação por *A. pleuropneumoniae*, e as vesículas extracelulares (VEs) produzidas podem mobilizar genes de resistência a antibióticos por vesidução.

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HIPÓTESE

Diferentes condições de estresse afetam a vesiculação de *A. pleuropneumoniae* e as VEs produzidas transferem marcadores de resistência a antimicrobianos por vesidução.

OBJETIVO GERAL

Caracterizar a produção de VEs em resposta a diferentes condições de estresse e avaliar a transferência do plasmídeo p780 por vesículas extracelulares (VEs) produzidas por *A. pleuropneumoniae* nestas condições.

OBJETIVOS ESPECÍFICOS

- Avaliar a curva de crescimento de *A. pleuropneumoniae* MV780 na presença e ausência de desferoxamina e de tetraciclina.

- Extrair e purificar VEs produzidas por *A. pleuropneumoniae* MV780 durante o crescimento, nas fases exponencial e estacionária, na presença e ausência de desferoxamina e tetraciclina.

- Caracterizar as VEs produzidas de *A. pleuropneumoniae* MV780 nas diferentes condições investigadas.

- Investigar a presença do plasmídeo p780 nas VEs produzidas nas condições investigadas acima.

- Avaliar a capacidade de transferência do plasmídeo p780 via VEs produzidas por *A. pleuropneumoniae* MV780 para outra linhagem de *A. pleuropneumoniae* não resistente.

MANUSCRIPT

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Vesiculation by *Actinobacillus pleuropneumoniae* is stimulated by different stress conditions and mobilizes antibiotic resistance genes by vesiduction

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Abstract

Aims

Extracellular vesicles (EVs) from *A. pleuropneumoniae* are affected by different stress conditions and EVs can have cargo as antimicrobial resistance genes and mobilize these markers by vesiduction. Here, we sought to investigate the production of EVs by *A. pleuropneumoniae* under various stress conditions and evaluated their ability to transfer the p780 (non-mobilizable tetracycline plasmid) through EVs.

Methods and results

EVs from *A. pleuropneumoniae MV780* grown under different stress conditions, such as iron depletion and tetracycline presence, were obtained through hydrostatic filtration, quantified, and characterized. Vesiduction assays were performed to evaluate the transfer of p780 to other clinical isolates from pig respiratory diseases. Our findings demonstrate that vesiculation is stimulated under conditions of low iron and in the presence of antibiotics in *A. pleuropneumoniae*. Under these conditions, plasmids can be transported via extracellular vesicles between isolates of *A. pleuropneumoniae*.

Conclusions

Our findings indicate that vesiculation is stimulated under conditions of low iron and in the presence of antibiotics, enabling the transport of plasmids between isolates of *A. pleuropneumoniae*. This explains the dissemination of plasmids among bacteria of the same habitat, such as swine respiratory pathogens.

Impact Statement

Identifying *in vitro* conditions that enhance EV production can drive significant biotechnological advances, enabling the use of these vesicles as vaccine platforms against swine pleuropneumonia, addressing substantial challenges in the swine industry.

Keywords: Extracellular vesicles. Porcine pleuropneumonia. Horizontal Gene Transfer – HGT. antimicrobial resistance.

Introduction

The global demand for pork is steadily increasing, driven by rising income and population growth in various regions worldwide, especially in Asia (YU et al., 2022). Due to this, global pork production has experienced rapid growth in recent decades. Several pathogens are capable of causing respiratory diseases in pigs, among them *Actinobacillus pleuropneumoniae*, the causal agent of swine pleuropneumonia, a disease already reported in almost all countries with intensive pig farming (STRINGER et al., 2021). *A. pleuropneumoniae*

is a Gram-negative, coccobacillary, facultative anaerobic, non-spore-forming, and non-motile bacterium (DONACHIE; LAINSON; HODGSON, 1995). Currently, 19 serotypes of *A. pleuropneumoniae* are known, which have been distinguished based on the antigenic properties of capsule polysaccharides (BOSSÉ et al., 2018; STRINGER et al., 2021). The occurrence of different biotypes and serotypes of *A. pleuropneumoniae* varies significantly across different global regions (SÁRKÖZI et al., 2018). In Europe, studies indicate that strains of serotype 2 predominate in specific areas, such as in Spain, where serotypes 2, 4, and 7 are more common (MALDONADO et al., 2009). In Switzerland, serotype 2 is also frequently reported (STÄRK et al. em 2007).

In the United Kingdom, predominant serotypes include 2, 3, 6–8, 10, 12, and 9 (DUBREUIL et al., 2000; O'NEILL et al., 2010). In Brazil, particularly in the state of Minas Gerais, the most prevalent serotypes of *A. pleuropneumoniae* are 5, 7, and 8, with serotype 8 being the most frequently reported (ROSSI et al., 2013; KUCHIISHI et al., 2023). In this context, the virulence of *A. pleuropneumoniae* is complex and multifactorial, involving various factors such as the production of capsule polysaccharides, lipopolysaccharides (LPS), toxins (Apx), iron-capturing proteins (siderophores), and the production of extracellular vesicles (EVs) (NEGRETE-ABASCAL et al., 2000; PEREIRA et al., 2018; CHIERS et al., 2010; FREY, 2011; SILVA et al., 2022; SILVA et al., 2023).

The ability of microorganisms to adapt and survive in diverse environments requires a robust ability to adjust and implement various mechanisms to ensure survival (HOSSEINI-GIV et al., 2022). In this context, the production of extracellular vesicles is an effective and complex process for each organism, demonstrating distinct responses to different environmental stimuli (TOYOFUKU et al., 2023). Extracellular vesicles (EVs) are nanoparticles formed by a lipid bilayer that play various roles, ranging from responding to environmental factors to communication between bacteria and interactions with host cells (TOYOFUKO et al., 2022). They have diameters ranging from 20 to 400 nm, showing differences in composition and charge (LIU et al, 2022).

The production of extracellular vesicles (EVs) is a ubiquitous mechanism in the three domains of life: *Bacteria*, *Eukarya*, and *Archaea* (GILL et al., 2019). Differences in the classification of bacterial EVs have been described, related to their biogenesis, release pathways, content, and function (TOYOFUKU et al., 2022). EVs in Gram-negative bacteria

were first identified in the 1960s when they were isolated from cell supernatants of *Escherichia coli* (ROTHFIELD, 1965). It is described that Gram-negative bacteria produce extracellular vesicles, with sizes ranging from 10 to 300 nm (JAN, 2017; TOYOFUKU et al., 2019; LIU et al., 2022).. Various conditions can induce the formation of EVs such as the presence of antimicrobials, nutrient deprivation, chelating agents reducing iron availability in culture media, pH alteration, temperature elevation, among others (ORENCH-RIVERA & KHUEN, 2016; ROIER et al., 2016; KIM et al., 2020; MOZAHEB & MINGEOT-LECLERCQ, 2020; CREWE, 2023). But, in *A. pleuropneumoniae*, it is not known what the impact of stress conditions is on the production of EVs. Iron acquisition is crucial for bacterial growth, metabolism, and colonization, being essential for the survival of most bacteria (COOK-LIBIN et al., 2022).

Actinobacillus pleuropneumoniae employs specific strategies, such as using porcine transferrin, hemoglobin, releasing extracellular vesicles, and externally supplied siderophores, to acquire iron from the cellular environment (NIVEN et al., 1989; BÉLANGER et al., 1995; DIARRA et al., 1996; BALTES et al., 2002; PEREZCHICA et al., 2023). In the context of EV production by App, the release of these nanoparticle has been reported, with various proposed biological functions, such as the transport and delivery of proteases and Apx toxins, transport of genetic material (sRNAs), and their potential use as a vaccine platform (NEGRETE et al., 2000; ANTENUCCI et al., 2018; ANTENUCCI et al., 2019; SILVA et al., 2022; SILVA et al., 2023).

In many countries, antimicrobial agents are commonly utilized in clinical practice to treat respiratory diseases (EKAKORO & OKAFOR, 2019). Within this context, membrane lipids and proteins emerge as critical targets for antibiotics (YANG et al., 2023). Studies demonstrate that extracellular vesicles act as a protective shield for bacteria against antibiotics (SCHWECHHEIMER & KUEHN, 2015; KIM et al., 2018), involving mechanisms of horizontal gene transfer (HGT), enzymatic inactivation, and direct degradation/sequestration of antibiotics (CIOFU, O. 2000; SCHAAR et al., 2011; FULSUNDAR et al., 2014; KULKARNI et al., 2015;). The function of EVs against antibiotics is not fully characterized, but there are indications that stressors, such as antibiotic treatment, stimulate the release of EVs by bacterial cells, as observed in *Escherichia coli, Shigella dysenteriae and Pseudomonas aeruginosa* (MACDONALD et al., 2013; BAUWENS et al., 2017; MICHEL et al., 2020; MICHEL et al., 2022; DHITAL, 2022).

Cunha (2015) identified a plasmid, p780, in an isolate of *A. pleuropneumoniae* from Minas Gerais, Brazil. The p780 plasmid consists of 5,129 bp and can be found in two versions: a larger one of 5.1 kb and a smaller version of 2.2 kb. The smaller version of this plasmid occurs due to recombination between the direct repeat sequences flanking the *tetB* gene (Supplementary Figure 1). In addition to the *tetB* gene, which encodes a protein responsible for tetracycline resistance, the plasmid also contains the *rep* gene, involved in plasmid replication. However, Cunha did not identify probable *oriT* and *Tra* region for p780. Subsequent studies elucidated that p780, despite not being mobilizable or conjugative, exhibited 100% similarity with a plasmid from *Pasteurella multocida* (pB1001) (MILLAN et al., 2009; LI et al. 2018). Several studies have documented the occurrence of Horizontal Gene Transfer (HGT) among different species, mediated by Extracellular Vesicles (EVs). These findings suggest a possible explanation for the dissemination of the plasmid p780 among distinct species.

Therefore, the main objective of this research is to characterize the production of extracellular vesicles (EVs) in response to stress conditions and to evaluate the sharing of genetic material mediated by vesicles in *A. pleuropneumoniae*, aiming for a better understanding of how the HGT of genes related to the application occurs.

Material and Methods

Bacteria strains and growth conditions

Actinobacillus pleuropneumoniae serotype 8 MV780 (Genbank accession number JSVV00000000.1) and *Actinobacillus pleuropneumoniae* serotype 8 MV597 (Genbank accession number JSVX00000000.1) were obtained through donation from the company Microvet - Veterinary Microbiology Special (Viçosa, Minas Gerais, Brazil). Both belong to the collection of animal pathogenic bacteria from the Bacteria Molecular Genetics Lab/Bioagro/UFV. The isolates were cultured on Brain Heart Infusion agar (BHI, BD - 237500) supplemented with Nicotinamide Adenine Dinucleotide (NAD – 10 $mg.mL^{-1}$) (Sigma-Aldrich-N0632) at 37 °C for 24 hours/ 5% CO_2 atmosphere. Subsequently, to obtain pre-inoculum was cultivated in Brain Heart Infusion broth (BHI - BD) supplemented with Nicotinamide Adenine Dinucleotide (NAD – 10 $mg.mL^{-1}$) (Sigma-Aldrich) at 37 °C for 24 hours in a 5% CO_2 atmosphere, with continuous shaking at 180 rpm in the specific conditions: i. iron depletion - use of desferoxamine - DFO (Oncoexpress - 7896261005082) 50 $\mu M.mL^{-1}$ and ii. use of tetracycline (Sigma-Aldrich - 60-54-8) at 16 $\mu g.mL^{-1}$.

Determination of growth curve in stress conditions

Actinobacillus pleuropneumoniae was inoculated on BHI-NAD agar (10 mg.mL^{-1}) and incubated overnight at 37 °C, 5% CO2 atmosphere. Subsequently, isolated App colonies were collected and transferred to 20 mL of BHI broth supplemented with NAD (10 mg.mL^{-1}), and incubated overnight at 37 °C, 180 rpm. A portion of this overnight culture (2%) was transferred to 60 mL of BHI-NAD medium and incubated at 37 °C, 180 rpm. The initial optical density ($O.D._{600}$) was adjusted to 0.1 and initially measured every 30 minutes for the first five hours and subsequently every 1 hour until reaching 16 hours of growth. Optical density ($O.D._{600}$) was read on a Ultrospec 10 cell density meter (Amersham Bionsciences, DE), using sterile BHI-NAD as blank. The experiment was conducted in biological triplicate. The growth curve was analyzed in the conditions: BHI/NAD; BHI/NAD/ desferoxamine - at a concentration of 50 μM.mL^{-1} and BHI/NAD/tetracycline at 16 μg.mL^{-1}.

Extracellular vesicles extraction and purification

The EVs obtained during this study were extracted and purified according to SILVA et al., 2023. The *Actinobacillus pleuropneumoniae* MV780 strain was cultivated on BHI-NAD agar at 37 and 5% CO_2 for 24 hours. Subsequently, some colonies were transferred to 50 mL of BHI-NAD and incubated overnight at 37 °C with continuous shaking at 180 rpm. An aliquot was then transferred to 600 mL of BHI-NAD, adjusting its $O.D._{600}$ to 0.1. DFO (50 μM.mL^{-1}) and tetracycline (16 μg.mL^{-1}) were added in different cultures. The bacteria suspensions were cultured for ~7 hours (late exponential phase) and ~16 hours (late stationary phase), respectively. After each time, the culture supernatant was obtained by centrifugation (5000 rpm for 30 min) and filtered through a 0.45 μm membrane (Millipore, Billerica, MA, USA-HAWP04700). The filtrate was then added to a 1000 kDa dialysis membrane (Biotech CE Tubing - Spectrum Labs - 15320682) wrapped in a glass column and incubated for two days at 4 °C. Subsequently, 300 mL of Phosphate-Buffered Saline (PBS) was added to wash the filtrate, followed by an additional 300 mL of PBS for a final wash. For the purification of the obtained EVs under the mentioned conditions, the filtrate was dialyzed in PBS (1X) with low agitation. After a total of 12 hours, the samples were filtered through a 0.22 μm membrane (Cole-Parmer, Vernon Hills, IL, USA - EW-06061-68) and concentrated using a 10 kDa Amicon column (Millipore, Billerica, MA, USA - UFC901008). A 10 μL aliquot was used to confirm the absence of contamination in the obtained EVs. Finally, the samples were stored at -20 °C.

EVs characterization

EVs Morphology by Transmission Electron Microscopy (TEM)

To assess the physical integrity and morphology of the obtained EVs, the purified samples were diluted serially and visualized by transmission electron microscopy (Zeiss EM 109) at the Center for Microscopy and Microanalysis (NMM-UFV) a facility of the Universidade Federal de Viçosa. In this analysis, aliquots of 10 µL of each dilution were applied to formvar-coated gold grids (Sigma, USA - TEM-FCF200AU). Subsequently, the samples were contrasted with 3% uranyl acetate for 1 minute.

EVs size by Dynamic Light Scattering (DLS)

The assessment of the size and polydispersity of EVs was conducted using the Dynamic Light Scattering (DLS) using the Zetasizer Nano ZS equipment (Malvern Instruments, United Kingdom). Data acquisition and analysis were performed using Malvern Zetasizer software, version 8.02, to determine the mean hydrodynamic diameter of each EVs solution in 1X PBS pH 7.0 (150 µg.mL-1). The parameters used were refractive index: 1.332 and viscosity: 0.9043. The measurements were conducted at a temperature of 25 ºC, with three runs for each sample, and the intensity-weighted mean diameter was calculated.

EVs protein profile

To verify the protein profile of EVs produced by *A. pleuropneumoniae* MV780, a 12% polyacrylamide gel stained with Coomassie Blue was utilized, as described by Sambrook 1990. The cell pellet, obtained from the same EV extraction experiment, was transferred to tubes containing Lysing Matrix B beads (MP Biomedicals, CA, USA). Subsequently, the Precellys® Evolution homogenizer equipment (Bertin Instruments) was used for two cycles of 30 seconds to promote cell lysis. Finally, the extract was centrifuged at 10,000 xg for 10 minutes, and the supernatant was collected for subsequent use. Aliquots of 30 µg/mL of EVs and their cognate cells were added to sample buffer (50 mmol.L^{-1} Tris-HCl, pH 6.8; 100 mmol.L^{-1} dithiothreitol; 2% SDS; 0.1% bromophenol blue; 10% glycerol) and heated at 100 °C for 10 minutes. Proteins were separated by 12% SDS-PAGE (Green & Sambrook, 2012).

EVs quantification

The conditions of the EVs obtained during the late exponential growth phase (~7h) subjected to iron depletion-induced stress using DFO (µM.mL^{-1}) and TET(16 µg.mL^{-1}) were evaluated. Additionally, the EVs obtained during the late stationary phase (~16h) were analyzed

under iron depletion stress conditions using DFO (50 μM.mL^{-1}) and TET (16 μg.mL^{-1}). The EV samples and their cognate cells were evaluated through a protein abundance quantification assay. We employed the Bradford reagent (Sigma-Aldrich, USA - 15946), based on the BSA standard curve (0.1 to 1.4 μg/mL), and the QuantiPro™ BCA Kit (Sigma-Aldrich, USA - QPBCA), utilizing the BCA with a Bovine Serum Albumin (Sigma-Aldrich, USA, 232-936-2) standard curve (0.5 to 30 μg/mL) for accurate quantification. For a more refined quantification, flow cytometry was employed. Twenty microliters of EVs were treated with DNase I (Promega, Madison, USA - M6101) (20 μg.mL^{-1}) at a final volume of 200 μL. The treated samples were stained with an equal volume of propidium iodide (20 μg.mL^{-1}) (Live/DeadTM - Thermo Fisher Scientific) and 3,3′-dioctadecyloxacarbocyanine perchlorate (20 μg.mL^{-1}) (Sigma-Aldrich, USA - 34215-57-1). In both conditions, the samples were incubated in the dark for 30 minutes at 37 ºC. EVs quantification was carried out using the BD Accuri C6 flow cytometer (Accuri Cytometers, Belgium) equipped with a laser sources (488 nm and 635 nm) that promotes emission through FL2 (585/40 nm), FL3 (>670 nm) and FL4 (675/25 nm, control) filters. Monoparametric and biparametric histograms were analyzed using the BD Accuri™ C6 software system.

Detection of p780 plasmid in EVs produced by *A. pleuropneumoniae*

The *Actinobacillus pleuropneumoniae* MV780 strain carries the p780 plasmid, which confers resistance to tetracycline with a Minimum Inhibitory Concentration (MIC) of (≥16 μg/mL), as described by Li et al. (2018). To investigate the presence of genes associated with plasmid p780, responsible for tetracycline resistance, in EVs produced by *A. pleuropneumoniae* MV780, we used the polymerase chain reaction (PCR) technique for the genes indicated in Table 1. EVs samples were treated with RQ1 RNase-Free DNase (Promega, USA - M6101), following the manufacturer's instructions. To verify the presence of the molecular marker of the p780 plasmid, we used the primer pair tetBF/tetBR. PCR reactions were carried out with 1.25 U of GoTaq DNA polymerase enzyme (Promega - M3001) in a final volume of 50 μL of buffer containing 1.5 mM MgCl2, 0.2 mM of each dNTP, and 0.2 μM of each primer. The samples were denatured at 95°C for 2 minutes, followed by 35 reaction cycles (95°C for 45 seconds, 45 seconds of annealing according to the Tm of each primer pair (Table 1), 72°C for 45 seconds), followed by a final extension step at 72°C for 5 minutes. Finally, the PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized in a UV transilluminator (Loccus, USA). Subsequently, inverse PCR (iPCR) was performed to detect the plasmid in the

EVs under the BHI/NAD/TET condition (16h). Using 1 U of Phusion® High-Fidelity DNA Polymerase (Thermo Scientific - M0530S) for a final volume of 50 µl, following the manufacturer's recommendations. The iPCR steps included an initial denaturation at 98 °C for 30 seconds, followed by 35 cycles of denaturation at 98 °C for 10 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 3 minutes. The iPCR product was analyzed by 1.2% agarose gel electrophoresis. Finally, the PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized in a UV transilluminator (Loccus, USA).

Table 1 – Oligonucleotides used in this work

Target Gene	Source	Primer identification	Sequence (5'->3')	Tm (ºC)	Amplicon size (bp)	Reference
tetB	p780	tetB780_F	CCGAAGTAGGGGTTGAGACG	62.8	374	Silva (2015).
		tetB780_R	TAAAGCGATCCCACCACCAG	63.2		
rep		rep780_F	GCTGGGTATCTTCTCGCACT	62.9	331	This study.
		rep780_R	ACATCAACGAGCAGATCAGAT	61.1		
tetA		tetAi_F2	TGGCTTTTCATTAGCGGGTCT	63.7	4762	
		tetAi_R2	GCTGAGGTGGTATCGGCAAT	63.6		

Vesiduction assay

The vesiduction assay developed in this study was developed based on the natural transformation protocol described by Bossé et al. (2004). To control the vesiduction process, extracellular vesicles from *A. pleuropneumoniae* MV780 (EVs-TET ~16h) were treated with RQ1 RNase-Free DNase (Promega, USA - M6101) to ensure that no exogenous DNA influenced the vesiculation process. Additionally, the solution EVs were plated on BHI/NAD medium as a control to confirm the absence of contaminants. The *A. pleuropneumoniae* MV597 clinical isolate, sensitive to tetracycline, was cultured in BHI-NAD broth for 2 hours until reaching an O.D. $_{600}$ of 0.50 ($1.10x10^8$ $CFU.mL^{-1}$). Subsequently, 20 µL of the culture was transferred to BHI-NAD plates, forming a dot approximately 10 mm in diameter. The plate was incubated at 37°C for 120 minutes. Later, 100 µL of EVs-TET (100 µg.mL-1) was added on top of the growing culture. After incubation at 37 °C for 24 hours, in a 5% CO2 atmosphere, bacterial cells were removed with a sterile inoculation loop and resuspended in sterile phosphate-buffered saline (PBS 1x). The suspension was plated on a BHI-NAD medium containing tetracycline at concentrations of 2, 4, 8, and 16 µg/mL; control plates did not contain

tetracycline. For isolates grown in selective media, a stability test was carried out for seven generations.

Confirmation of Transvesiductants by PCR

To confirm the presence of p780 in the EVs to the recipient cell, a PCR was performed for the molecular markers *tetB* and *rep*, respectively. The markers are present in the p780 plasmids, indicating plasmid transfer. To verify vesiduction with the p780 plasmid, the primer pairs *tetBF/tetBR* and *repF/repR* (Table 1) were used. As a positive control, a sample of genomic DNA from the MV780 isolate was used; as a negative control, genomic DNA from the MV590 isolate (recipient) was used. PCR reactions were carried out with 1.25 U of the GoTaq DNA polymerase enzyme (Promega - M3001) in a final volume of 50 μL of buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.2 μM of each primer. The samples were denatured at 95 °C for 2 minutes, followed by 35 reaction cycles (95°C for 45 seconds, 45 seconds of annealing according to Tm of each primer pair (Table 1), 72°C for 45 seconds), followed by a final extension step at 72°C for 5 minutes. Finally, the PCR products were subjected to 1.2% agarose gel electrophoresis, visualized on a UV transilluminator (Loccus, USA).

Statistical Analysis

For the phenotypic and EV production analysis, differences were analyzed using analysis of variance (ANOVA), followed by the Tukey test for multiple comparisons. p-values < 0.05 were considered statistically significant. This analysis was performed using GraphPad PRISM® software Version 8.4.3 (San Diego, CA, USA).

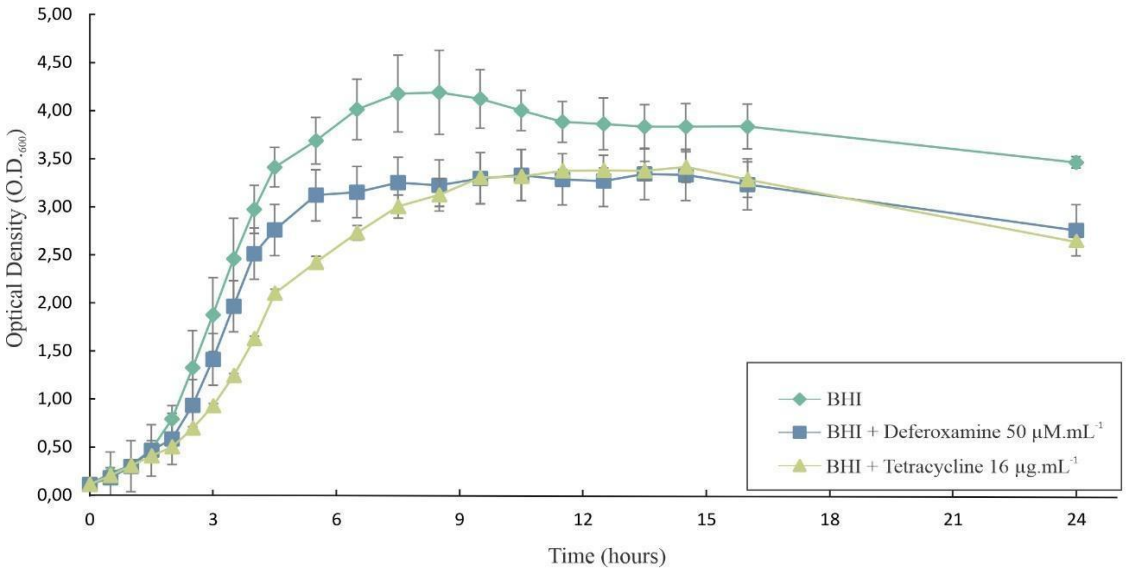
Results

Phenotypic Growth Analysis

To perform the extraction of EVs under stress conditions, we determined the growth curve and the maximum growth rate μ (h^{-1}) of isolate MV780 with and without the presence of stressors, DFO and TET, respectively. After 24 hours, we observed significant differences in the growth profile with or without the addition of DFO and TET (Figure 1A). The growth profile in the logarithmic phase was similar between the condition without the stressor and the condition with DFO added to the culture. However, in relation to the culture with tetracycline added to the medium, the growth was different, with slower exponential growth. We also analyzed the stationary phase, with the growth profile of isolate MV780 without the addition

of stressors, which differed in relation to the studied conditions. The profile in the stationary phase of the BHI/NAD/DFO and BHI/NAD/TET conditions was similar. The growth rate was calculated (Figure 1B), and the obtained values were subjected to ANOVA and Tukey's test. The statistical tests indicate that there is no significant difference between the BHI/NAD and BHI/NAD/DFO conditions, only when comparing the BHI/NAD and BHI/NAD/TET conditions (p-value < 0.05).

A.



B.

Organism	Conditions	Growth rate µ (h^{-1})
App MV780	BHI/NAD	0,7415
	BHI/NAD/desferoxamine (50 µM.mL^{-1})	0,657
	BHI/NAD/tetracycline (16 µg.mL^{-1})	0,427

Figure 1: Growth profile of *A. pleuropneumoniae* MV80 in different conditions. (**A)**The growth curves of *A. pleuropneumoniae* MV80 were analyzed under different culture conditions: BHI/NAD; BHI/NAD/deferoxamine (50 µM.mL^{-1}); BHI/NAD/tetracycline (16 µg.mL^{-1}) at 37 °C, under aerobic conditions. (**B).** Specific Growth Rate of *A. pleuropneumoniae* MV780 in Different Culture Conditions.

For this study, the EVs of *A. pleuropneumoniae* MV780 were extracted during two phases of bacterial growth. During the late exponential phase in BHI/NAD medium (~7h, O.D. $_{600}$ ~ 4.01 and ~3.15 × 10^9 CFU.mL^{-1}), in BHI/NAD/DFO 50 µM.mL^{-1} medium (~7h, O.D. $_{600}$ ~ 3,15 and ~4.7 × 10^8 CFU.mL^{-1}), and in BHI/NAD/TET 16 µg.mL^{-1} medium (~7h, O.D. $_{600}$ ~

2.73 and ~1.1 × 10^8 CFU.mL^{-1}). Finally, during the stationary phase under the conditions: BHI/NAD (~16h, O.D. $_{600}$ ~ 3.83 and ~1.5 × 10^{10} CFU.mL^{-1}), BHI/NAD/DFO 50 μM.mL^{-1} (~16h, O.D. $_{600}$ ~ 3.28 and ~2.1 × 10^9 CFU.mL^{-1}), and BHI/NAD/TET 16 μg.mL^{-1} (16h, O.D. $_{600}$ ~ 3.23 and ~1.35× 10^9 CFU.mL^{-1}).

Vesiculation by *A. pleuropneumoniae* in different conditions of stress

Morphology of EVs *A. pleuropneumoniae*

The EVs produced by *A. pleuropneumoniae* cultivated in BHI/NAD, BHI/NAD/DFO, and BHI/NAD/TET media at different growth stages were analyzed by transmission electron microscopy (TEM) to confirm their integrity and visualize structural characteristics. The EVs obtained in this work exhibited a heterogeneous profile, featuring a circular morphology with electron-dense content, and the presence of a double membrane (internal and external) was observed (Figure 2A - B). Our study demonstrated that *A. pleuropneumoniae* produces extracellular vesicles (EVs) in both growth phases, with a notable increase in the stationary phase. Under different stress conditions, these EVs consist mainly of outer membrane vesicles (OMVs), with a smaller population identified as outer-inner membrane vesicles (OIMVs)(Figura 2 A-B). Although we did not evaluate the specific proportion of each type of EV produced under different conditions, it is important to note that this proportion may vary. Transmission electron microscopy analyses revealed the presence of a double membrane in the EVs, indicating a distinctive structure.

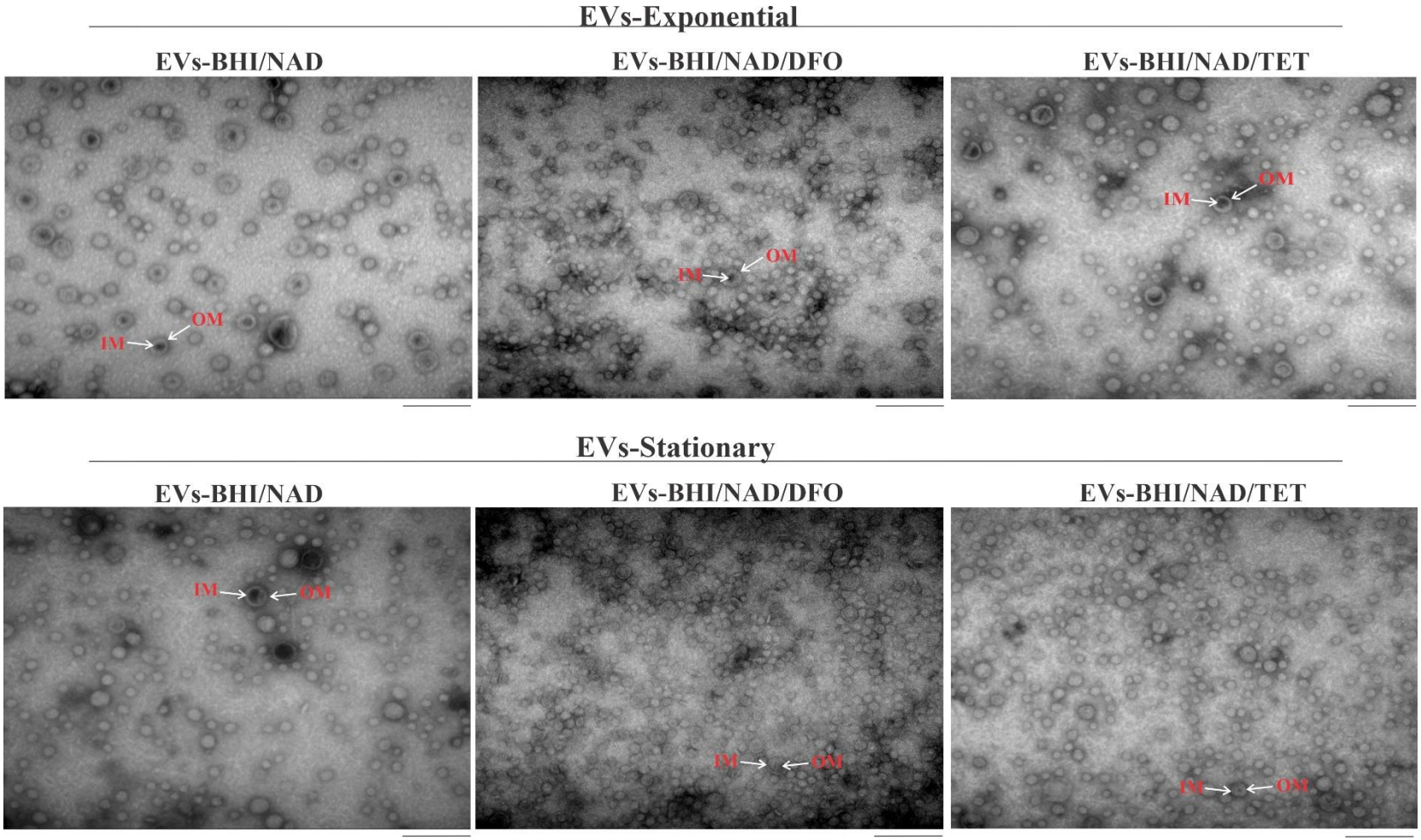


Figure 2: Morphology of EVs produced by *A. pleuropneumoniae* MV780 in different conditions - stressor condition and time. Transmission electron microscopy (TEM) of EVs extracted during the late exponential phase (7h) and the late stationary phase (16h). The abbreviations OM and IM refer to the outer membrane and inner membrane, respectively. All scale bars represent 200 nm.

A. pleuropneumoniae EVs size profile

The EVs produced by *A. pleuropneumoniae* under different culture conditions were different in size and dispersion profiles (Figure 3 A and B). During the late exponential growth phase (Figure 3 A), the EVs produced under different culture conditions showed variations in size and dispersion. EVs obtained under the condition without the addition of a stress agent were smaller, with a size range of 27 to 116.6 nm and a peak at 31.02 nm (~12%). On the other hand, EVs cultured under BHI/NAD/DFO and BHI/NAD stress conditions were larger, with sizes ranging from 27.16 to 187.5 nm and peaks at 49.24 nm (~20%) and 47.02 to 333.33 nm with a peak at 56.58 nm (~17.5%), respectively. The same pattern was observed in EVs obtained during the stationary phase (Figure 3 B), with an average size ranging from 20.47 to 106.2 nm and a peak at 25.79 nm (~11.5%) for EVs obtained under the condition without stressor. This differs from the profile of the EVs produced under both stress conditions, BHI/NAD/DFO and BHI/NAD, which exhibit a size range of 28.28 to 199.8 nm with a peak at 51.57 nm (~12.5%) and an average range of 44.89 to 328.4 nm with a peak at 55.56 nm (~10.3%), respectively

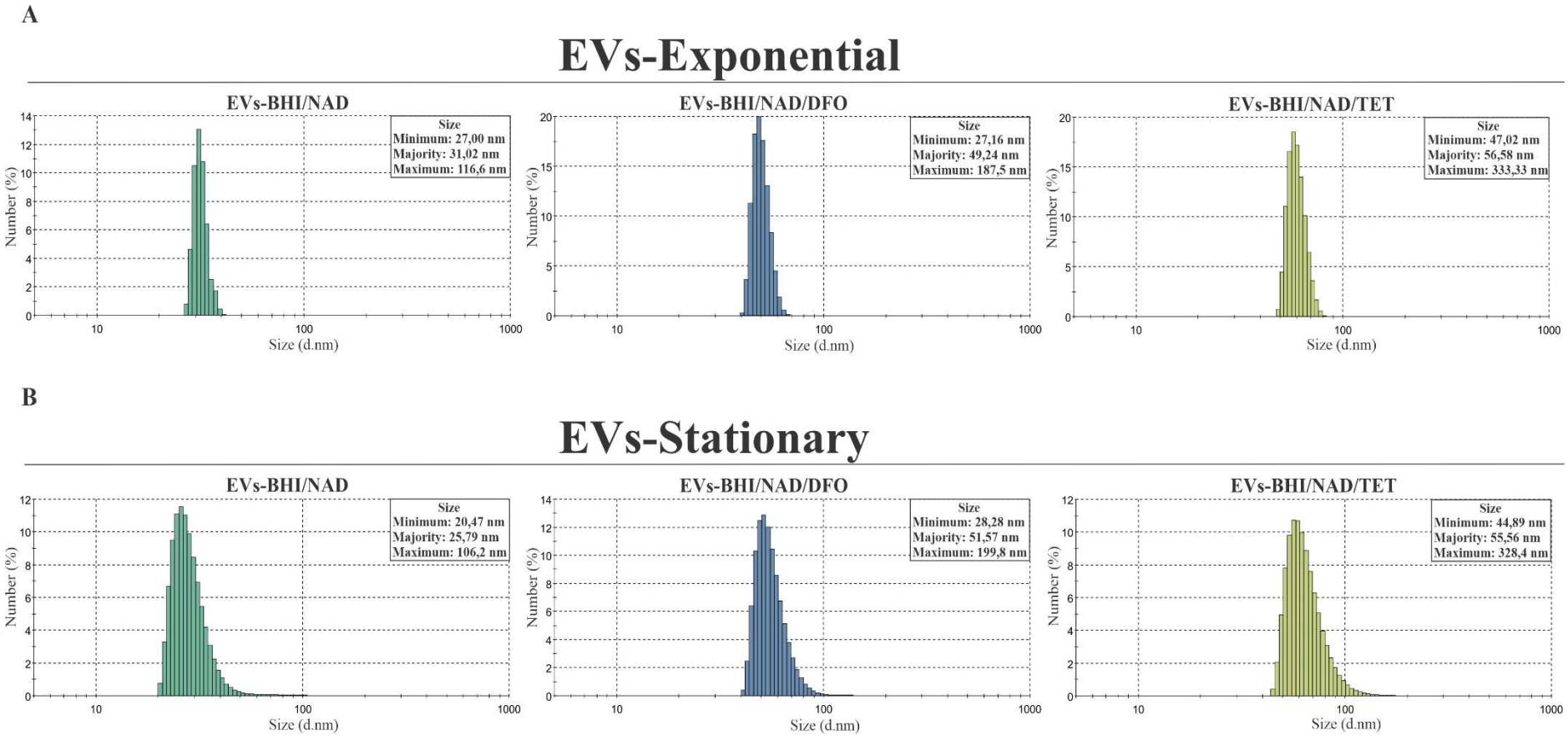


Figure 3: Size and Dispersion of EVs produced by *A. pleuropneumoniae* under different growth conditions.**(A)** Measurement of size and dynamic light scattering (DLS) profile of vesicles produced by *A. pleuropneumoniae* extracted during the late exponential phase (~7h) under different studied conditions.**(B)** Measurement of size and dynamic light scattering (DLS) profile of vesicles produced by *A. pleuropneumoniae* extracted during the stationary phase (~16h) under different studied conditions. The minimum, maximum, and most representative size in the EV samples are represented in the box in the upper right corner of each figure.

Protein profile of EVs produced by *A. pleuropneumoniae*

The protein profiles of EVs produced in the exponential growth phase (Fig. 4A) were similar to each other, featuring proteins ranging from 25 kDa to 255 kDa in size, differing only from the protein profile of their corresponding cells, which have a higher protein load. In the stationary phase (16h), the protein profile of EVs remained consistent under BHI/NAD and BHI/NAD/DFO conditions, with few observed variations (Fig. 4 B). A variation in the protein profile of the BHI/NAD/TET condition was observed and requires further study. Differences were noted between the profiles of EVs and their cells, which naturally exhibit distinct profiles, as expected.

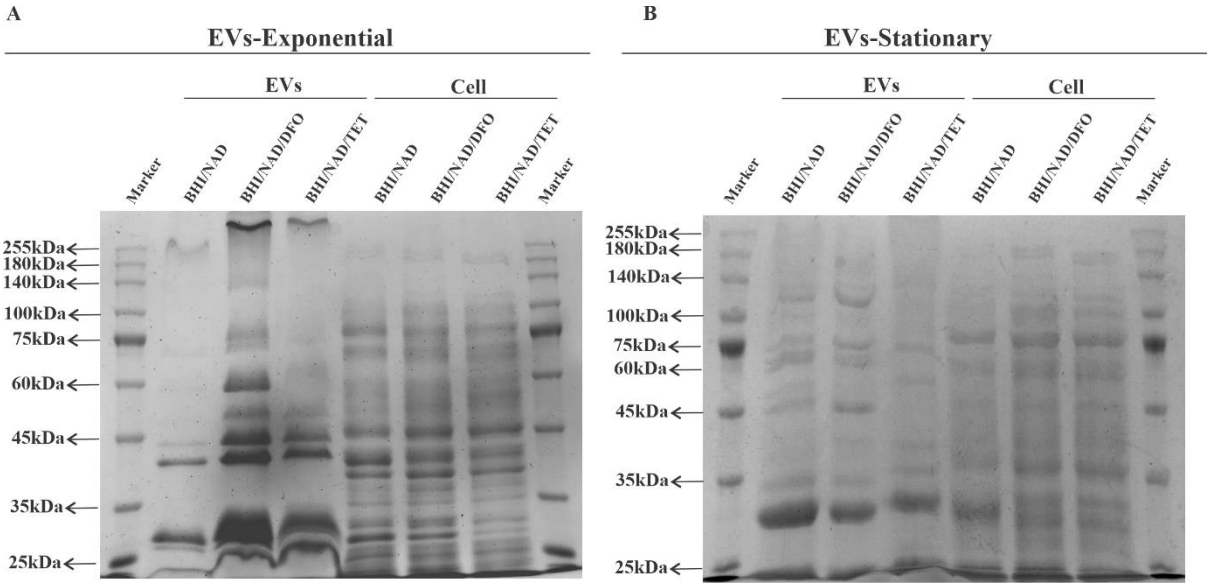


Figure 4: Protein profile of EVs and whole cells of *A. pleuropneumoniae* in different conditions of growth. **(A)** Protein profile of EVs extracted during the exponential growth phase (7h) and total cell extract at the same time. **(B)** Protein profile of EVs extracted during the stationary growth phase (16h) and total cell extract at the same time.

Production of EVs

For the protein quantification of EVs produced by *A. pleuropneumoniae* in the exponential growth phase (~7h), the Bradford reagent (Fig. 5 A) and the QuantiPro™ BCA Kit (Fig. 5 B) were employed. Both tests indicated a higher production of extracellular vesicles under iron-depletion stress conditions (0.607 $mg.mL^{-1}$ and 5.14 $mg.mL^{-1}$, respectively) by adding desferoxamine to the culture medium. The production profile remained consistent in EV quantification by flow cytometry using the dyes 3,3′-dioctadecyloxcarbocyanine perchlorate and propidium iodide (Fig. 5 C-D), where a higher production of EVs was observed in the BHI/NAD/DFO condition (12,418 EVs per mL and 308.6 EVs per mL).

A greater production of EVs was reported in the BHI/NAD/TET condition (10,423 EVs per mL and 184 EVs per mL) compared to the BHI/NAD condition. Regarding EVs extracted in the stationary phase (~16h), protein quantification using both the Bradford reagent (Fig. 6 A) and the QuantiPro™ BCA Kit (Fig. 6 B) indicated a higher production of EVs in the BHI/NAD/DFO condition (1.04 $mg.mL^{-1}$ and 10.336 $mg.mL^{-1}$, respectively) compared to the BHI/NAD and BHI/NAD/TET conditions.

The Bradford test also indicated a higher production of EVs in the BHI/NAD/TET condition (0.7455 $mg.mL^{-1}$) compared to the BHI/NAD condition ($mg.mL^{-1}$), a result not corroborated by the QuantiPro™ BCA Kit, where a higher concentration of EVs in the BHI/NAD condition (5.61 $mg.mL^{-1}$) was found compared to the BHI/NAD/TET condition (5.54 $mg.mL^{-1}$). Flow cytometry quantification using the dyes 3,3′-dioctadecyloxcarbocyanine perchlorate and propidium iodide (Fig. 6 C-D) revealed a higher production of EVs in the BHI/NAD/DFO condition (21,752 EVs per mL and 578 EVs per mL) compared to the BHI/NAD conditions (16,818 EVs per mL and 364 EVs per mL) and BHI/NAD/TET condition (21,752 EVs per mL and 463 EVs per mL).

A normalization was performed to verify the proportion of EVs (quantified by flow cytometry/CFU.mL), showing that significantly more EVs were produced in the BHI/NAD/TET condition for both extraction conditions (late exponential and late stationary phases) (Fig. 5 E) and (Fig 6 E). Significant differences between the means of the studied conditions are represented by different letters after comparisons, as determined by t-tests (p-values < 0.05). Equal letters mean that no statistical difference was found.

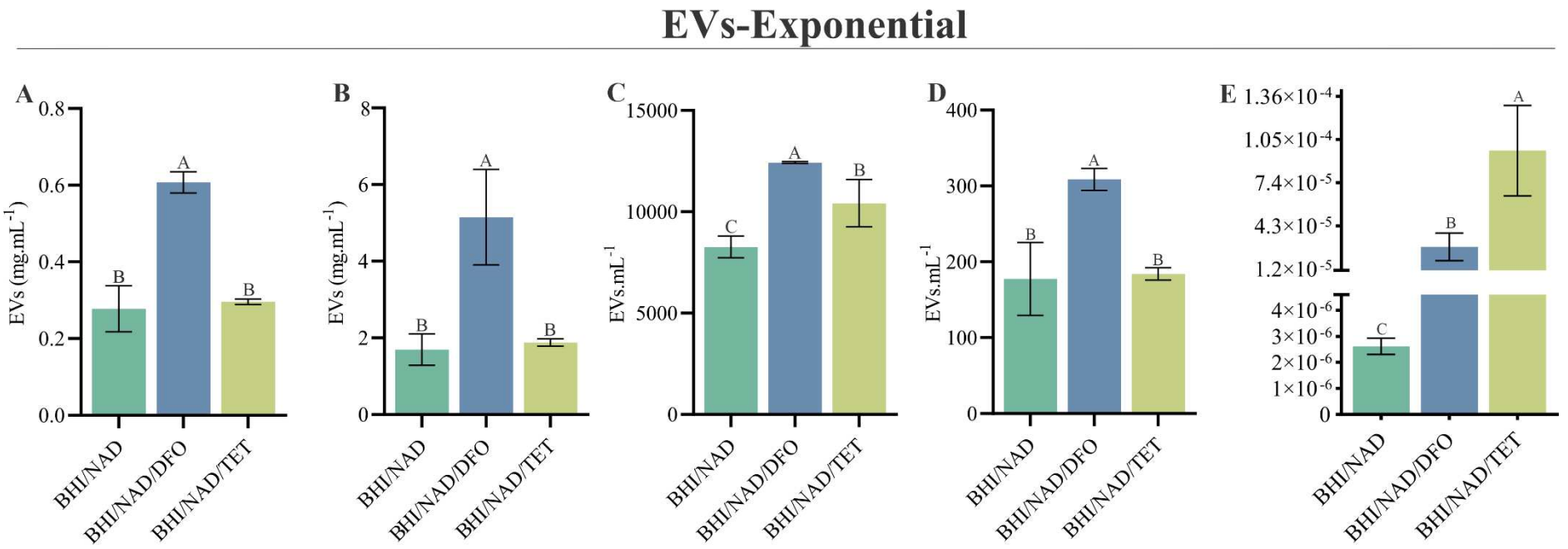


Figure 5: Production of EVs by *A. pleuropneumoniae* MV780 under diferent conditions. **(A)** and **(B)** Protein quantification of EVs. **(C)** and **(D)** Quantification of EV numbers by flow cytometry. **(E)** Normalization of EV production in the exponential phase by flow cytometry**.** Significant differences between EV production are indicated by different letters, and equal letters indicate no significant difference between means, as calculated by t-tests (p-values < 0.05).

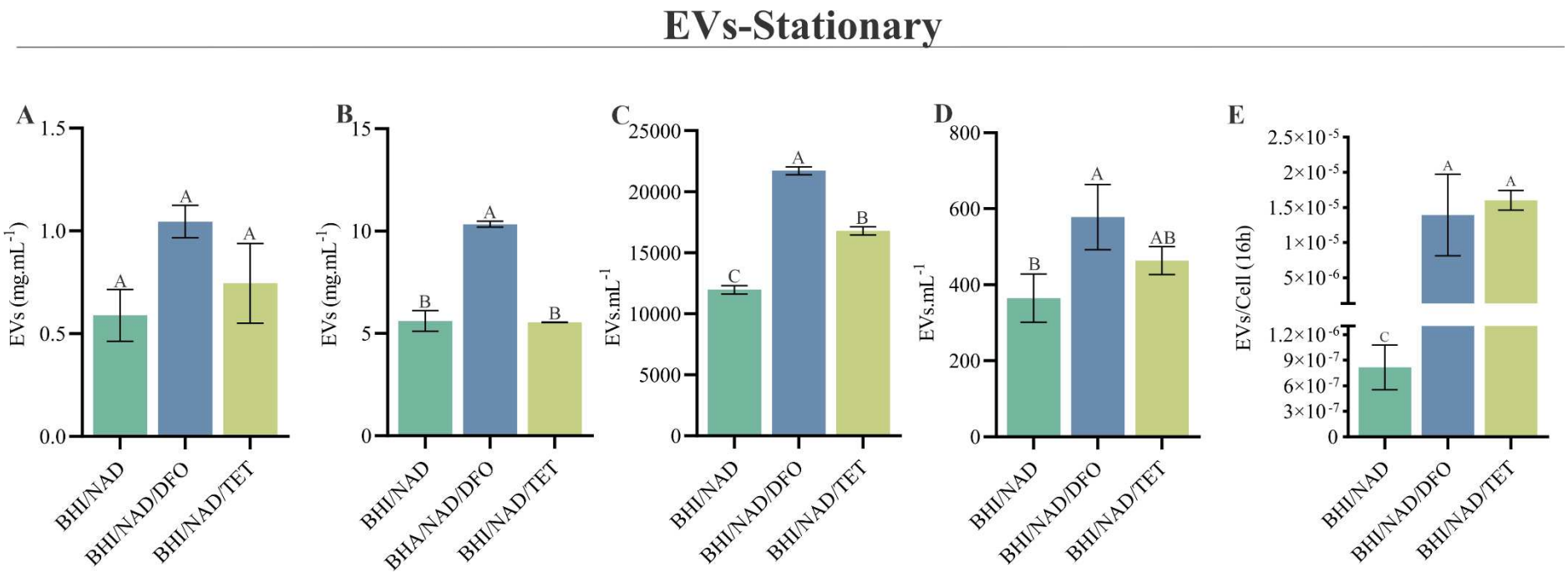
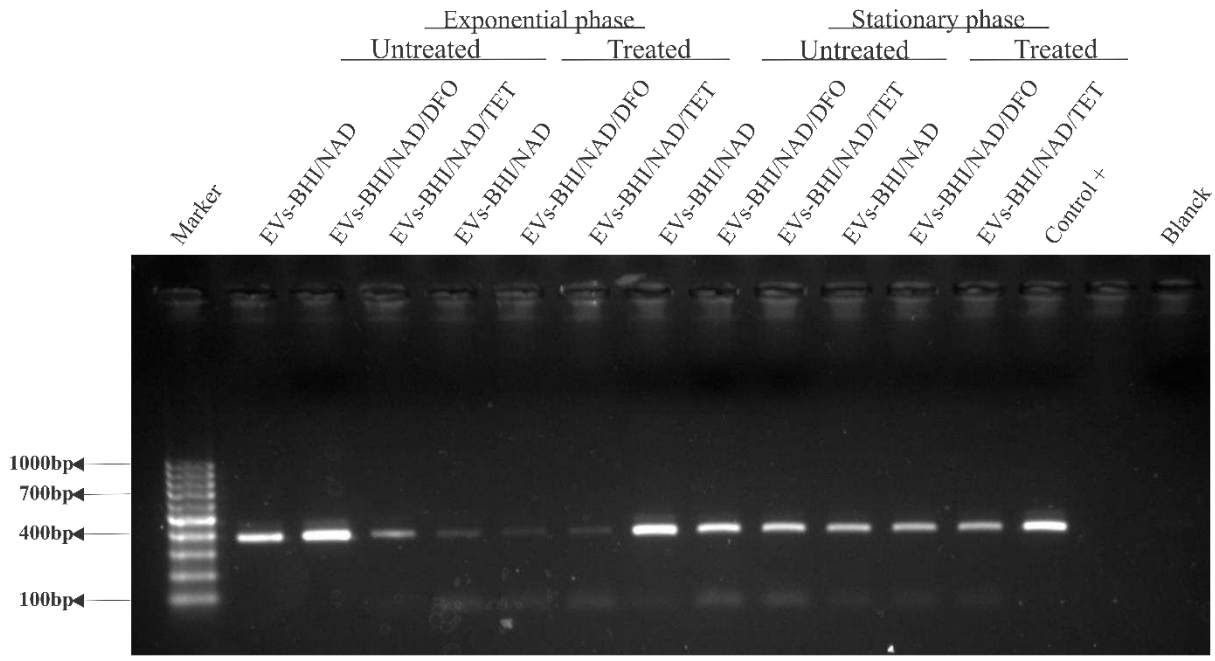


Figure 6: Production of EVs by *A. pleuropneumoniae* MV780 under stress conditions. **(A)** and **(B)** Protein quantification of EVs. **(C)** and **(D)** Quantification of EV numbers by flow cytometry. **(E)** Normalization of EV production in the stationary phase by flow cytometry. Significant differences between EV production are indicated by different letters, and equal letters indicate no significant difference between means, as calculated by t-tests (p-values < 0.05).

Detection of the markers genes from p780 inside *A. pleuropneumoniae* EVs

After characterizing the EVs produced by *A. pleuropneumoniae* in different growth phases and under different stress conditions, we investigated the presence of molecular markers for the p780 plasmid in all the investigated conditions. The p780 plasmid has been previously characterized and sequenced in Silva (2015) undergraduate thesis. In App, the p780 plasmid confers resistance to tetracycline with a minimum inhibitory concentration (MIC) of (≥16 $\mu g.mL^{-1}$). To confirm that *A. pleuropneumoniae* EVs carry the p780 plasmid, a polymerase chain reaction (PCR) for the *tetB* gene was performed. The EVs were then treated with DNase I (20 $\mu g.mL^{-1}$) to demonstrate that the *tetB* gene is packaged by EVs and protected from degradation by endonucleases. The presence of this molecular marker was detected in all EVs in this study (FIG. 7 A). As it is directly related to tetracycline resistance, and for a better description of the possible presence of p780 in App EVs, EVs from the BHI/NAD/TET condition (~16h) were chosen to confirm the presence of both versions (5.1 kb and 2.2 kb) of the closed p780 carried by the EVs. The presence of both versions of p780 in EVs-BHI/NAD/TET was confirmed through inverse polymerase chain reaction (iPCR) (FIG. 7 B).

A



B

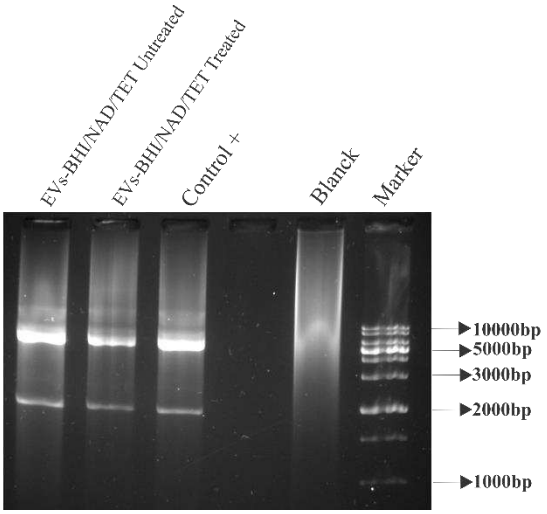


Figure 7: **(A)** Plasmid detection in *A. pleuropneumoniae* EVs. Detection of the presence of the *tetB* resistance gene in all EVs obtained in this study, treated and not treated with DNase I. Cellco marker 100 bp. **(B)** Detection of the two versions of the p780 plasmid in EVs-BHI/NAD/TET. Kasvi 1Kb size marker. The positive control for the PCR and iPCR was the genomic DNA of *A. pleuropmeumoniae* MV780.

Vesiduction acting on Horizontal Gene Transfer (HGT)

Subsequently, we examined the potential of EVs acting as a mechanism for horizontal gene transfer between *A. pleuropneumoniae* isolates. To ensure this, we ensured that the donor strain did not exhibit tetracycline resistance genotypes and phenotypes. Both App MV780 and App MV597 were used as positive controls in a medium containing only BHI/NAD (FIG. 8 A). *A. pleuropneumoniae* MV597 does not grow at any tetracycline concentration; a control was performed at the lowest concentration used in the vesiduction assay (2 µg.mL^{-1}), as shown in (FIG. 8 B). In this study, we used tetracycline concentrations of 2, 4, 8, and 16 µg.mL^{-1} for the selection of transvesiductants. As a result, growth was observed only in plates containing 2 µg.mL^{-1} of tetracycline in the culture medium (FIG. 8 C). The transvesiductants showed growth in TET at 2 µg.mL^{-1} for more than 7 generations, indicating a stable phenotype. Finally, total DNA extraction was performed from the transvesiductant cells for the detection of molecular markers for the p780 plasmid (FIG. 8D).

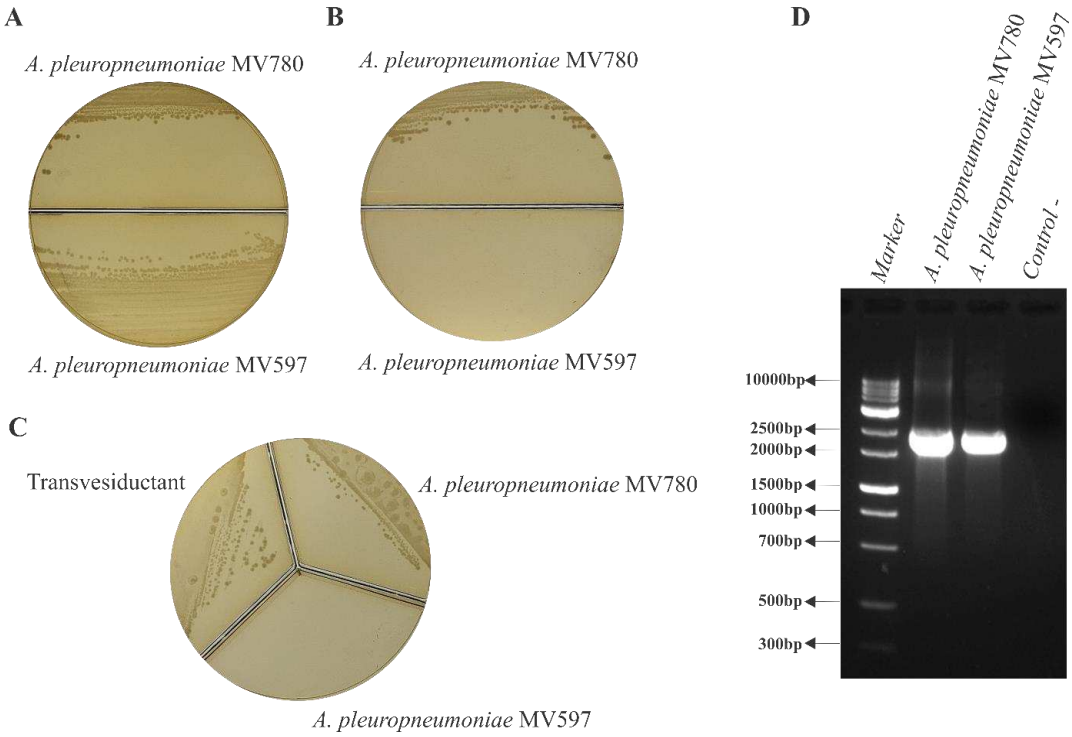


Figura 8: Phenotypes and genotypes presented by the transvesiductants.**(A)** Growth of App 780 and 597 isolates in BHI/NAD without addition of tetracycline. **(B)** Growth phenotype of the App MV597 isolate on App MV597 BHI/NAD agar plus tetracycline (2 µg.mL^{-1}). **(C)** Growth phenotype of the transvesiductant on BHI/NAD agar plus tetracycline at a concentration of (2 µg.mL^{-1}). **(D)** Detection of molecular markers for the p780 plasmid. KASVI 1Kb size marker.

Discussion

For the first time, we demonstrated that *A. pleuropneumoniae* releases extracellular vesicles (EVs) during different growth phases and under distinct stress conditions. The stress can be induced either by the low availability of iron ions, through the action of the chelator desferoxamine (DFO), and by the use of antimicrobial agents, such as tetracycline (TET), added to the culture medium. In addition to characterizing the EVs produced under stress conditions, we assessed the potential for horizontal gene transfer (HGT) through extracellular vesicles, representing the first report of the vesiduction in *Actinobacillus pleuropneumoniae*.

Here, we demonstrate that vesiduction may be the primary mechanism involved in the transfer of non-mobilizable plasmids containing antimicrobial resistance genes among pathogenic animal bacteria. This could explain the co-occurrence of plasmid pB1001 from *Pasteurella multocida*, which confers resistance to tetracycline and was first described in 2009, being found in a clinical isolate of *A. pleuropneumoniae* in the state of Minas Gerais, Brazil, in 2015, also conferring resistance to tetracycline (MILLAN et al., 2009; LI et al. 2018). In studies by Liu and colleagues (2018), it was evidenced that plasmid p780 is neither a conjugative nor mobilizable plasmid, which would prevent its transfer through traditional methods of horizontal gene transfer. The presence of identical plasmids in multiple species of the *Pasteurellaceae* family suggests a mechanism of horizontal transfer that does not involve the conjugative apparatus, thus showing that species sharing the same niche (respiratory tract of pigs) can transfer genetic elements via EVs.

Animal health faces numerous challenges, including complex infectious diseases and antimicrobial resistance (VELAZQUEZ-MEZA et al., 2022). In this context, extracellular vesicles may be the key to a better understanding of bacterial pathogenesis, as we have proven that stress conditions such as iron depletion and the presence of antimicrobials stimulate the production of EVs. Previous studies already report that bacteria under different stress conditions can increase their capacity for EV production (ROSALES, 2014; CHAN et al., 2016; LIN et al., 2017; VOLGERS et al., 2017; YUN et al., 2018; ZINGL et al., 2020. Thus, EVs produced under a stress condition can carry content that expands the area of lesions peculiar to the disease, as in the case of porcine pleuropneumonia, and can also favor the THG of markers of resistance to antimicrobials and virulence among bacteria from the same habitat, such as that cause respiratory diseases in pigs. Studies indicate that EVs secreted by *A. pleuropneumoniae* can act as important vectors of virulence, inducing an increase in the strain's virulence capacity (SILVA et al., 2022).

Regarding the size of released EVs, there was an increase in the size of these vesicles for all stress conditions, either by DFO or TET, in both growth phases, exponential and stationary, respectively (Figure 3). This increase in the average size of EVs is already described for other bacteria, such as in the case of *N. gonorrhoeae*, where the absence of iron ions leads to an increase in the average diameters of OMVs secreted under iron deprivation conditions (PŁACZKIEWICZ et al., 2023). In *Staphylococcus aureus*, a significant increase in the secretion of extracellular vesicles and an increase in the average size of secreted EVs occur under ampicillin stress conditions (KIM, S. et al., 2020). An important point to discuss is that the presence of antimicrobial resistance plasmids can also induce an increase in the average size of produced EVs. Tran & Boedicker (2017) showed that the presence of plasmids in *E. coli* EVs can affect the size of these secreted vesicles. In our study, EVs produced by App carry a plasmid that confers resistance to tetracycline (Figure 7), which may indicate one of the reasons for the increase in EVs under stress conditions.

In our study, we have demonstrated that vesiculation in *A. pleuropneumonae* is stimulated under stress conditions in both the exponential and stationary phases, respectively. The quantification of EVs in this work revealed a higher production of EVs under iron deprivation stress conditions. However, after normalizing the production of EVs per $CFU.mL^{-1}$, *A. pleuropneumonae* cultivated under tetracycline stress produced more EVs than under iron deprivation stress in both growth phases (Figure 5-6). Protein extraction was conducted to characterize the protein profile of extracted extracellular vesicles (EVs). EVs produced in the exponential phase exhibited a protein profile ranging from 255 kDa to 25 kDa. Differences in band profiles were observed when comparing stress conditions with DFO and TET to growth without any stressor. In EVs produced in the stationary phase, the condition with the addition of DFO showed differences compared to BHI/NAD and BHI/NAD/TET conditions, highlighting variations in protein abundance between sizes of 75 kDa to 45 kDa (Figure 4).

In our study, we reported for the first time the presence of an intact plasmid being released through EVs in different growth phases and stress conditions in App. Resistance genes belonging to the p780 plasmid (5.1 kb) were found within App EVs, indicating the presence of this plasmid within the vesicles (Figure 7 A).It is already described that EVs have the ability to protect DNA and RNA from external factors such as DNases, restriction enzymes, and physical and chemical agents (YÁÑEZ et al., 2015). It has been described that small RNAs (sRNAs) are secreted via EVs in *A. pleuropneumoniae* (SILVA et al., 2023). Through EVs, plasmids can be transferred between cells (Tran & Boedicker, 2019). The p780 plasmid has been previously

characterized by Silva (2015) and has two versions, one of 5.1 kb and a second version of 2.1 kb. Through inverse polymerase chain reaction (iPCR), we detected the presence of both versions of the plasmid in EVs from App under tetracycline stress conditions (Figure 7 B).

Currently, there is no research on horizontal gene transfer (HGT) via EVs in *A. pleuropneumoniae*. Here, we conducted, for the first time, a vesiduction experiment to test HGT mediated by EVs from the *A. pleuropneumoniae* MV780 isolate. As a result, we observed that the MV597 strain grew in BHI/NAD medium supplemented with TET (2 μg/mL), a phenotype obtained after vesiculation and maintained by cells for 7 generations (Figure 8 C). Finally, we confirmed the presence of a molecular marker for this plasmid in the recipient cells (Figure 8 D). EVs can serve as a delivery system for plasmids and genes conferring resistance to numerous antibiotics (CHATTERJEE et al., 2017). There are reports of various species performing Horizontal Gene Transfer (HGT) through Extracellular Vesicles (EVs). Published studies have documented the transfer of penicillin resistance genes in *Neisseria gonorrhoeae* and an oxacillinase in *Acinetobacter baumannii* (RUMBO et al., 2011; JIN et al., 2011). In *E. coli*, the transfer of plasmids up to 15 kb to recipient cells has been observed, with plasmid characteristics influencing the exchange of genetic material with the recipient cell (TRAN & BOEDICKER, 2019).

In conclusion, the results obtained in this study highlight, for the first time, that stress conditions, whether due to iron deprivation or stress mediated by antimicrobial agents, can induce vesiculation in an isolate of *A. pleuropneumoniae*, a significant pathogen causing numerous losses in the swine industry. Furthermore, we report that a plasmid conferring tetracycline resistance in App can be internalized in EVs produced by this pathogen and transferred through a novel mechanism of horizontal gene transfer to another isolate of tetracycline-sensitive *A. pleuropneumoniae*. Our study not only demonstrates that stress conditions stimulate the production of EVs by bacteria but also suggests that this new mechanism may contribute to the transfer of genetic material between isolates of the same species, thus contributing to genetic diversity.

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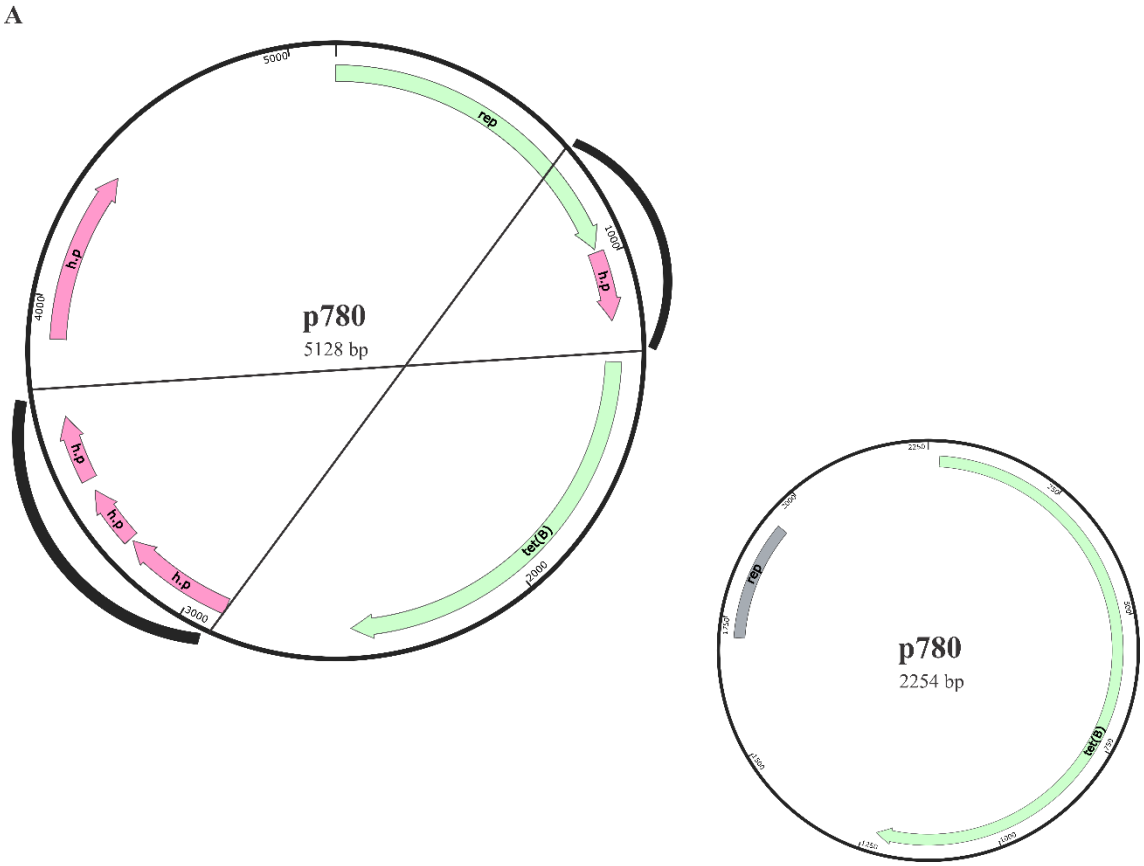
Author contributions

All authors contributed to the conception and structuring of this study. M.G, G.S, J.N and P.F obtained and analyzed the data. W.C assisted in the experimentation and interpretation of flow cytometry. D.B, H.M, and P.L helped in writing and correcting the article. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no competing interests.

Supplementary material



Supplementary Figure 1: Schematic representation of p780. The larger version (5128 bp) and smaller version (2253 bp) found in *A.pleuropneumoniae*. Its smaller version is formed by a recombination between repeat sequences (shown as black bands) flanking the *tet*(B) gene, as indicated by the crossed lines connecting the ends of the repeated sequences in p780. In the larger version, the pink arrows represent genes described as hypothetical proteins, while the green one corresponds to the *tetB* and *rep* gene. In the smaller version, the green arrow corresponds to the *tet*B gene, and the gray represents the truncated part of the rep gene after recombination.

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Conclusão

Este estudo representa o primeiro relato na qual comprova que condições de estresse, resultantes tanto da escassez de ferro quanto da exposição a agentes antimicrobianos, aumentam

a produção de VEs em *A. pleuropneumoniae*. Esta bactéria é um patógeno de significativa relevância na suinocultura, responsável por causar consideráveis prejuízos econômicos. Além disso, observamos pela primeira vez em *A. pleuropneumoniae* que um plasmídeo, anteriormente descrito e possivelmente disseminado entre espécies da família *Pasteurellaceae*, conferindo resistência à tetraciclina, pode ser transportado dentro de VEs e transferido por meio de um novo mecanismo de transferência horizontal de genes (HGT), denominado de vesidução, para outro isolado de *A. pleuropneumoniae* suscetível à tetraciclina.

A identificação de condições *in vitro* que aumentam a produção de VEs representam passos importantes e primordiais que impulsionam avanços biotecnológicos significativos, possibilitando o uso dessas vesículas como plataformas de vacinação no combate a doenças respiratórias em suínos, especialmente contra a pleuropneumonia suína.