UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS AGRÁRIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

Daiane Voss Rech

IMPACTO DE TRATAMENTOS DE CAMA AVIÁRIA REUTILIZADA NA VIABILIDADE E INFECTIVIDADE DE MICRO-ORGANISMOS

Santa Maria, RS
2017

Daiane Voss Rech

IMPACTO DE TRATAMENTOS DE CAMA AVIÁRIA REUTILIZADA NA VIABILIDADE E INFECTIVIDADE DE MICRO-ORGANISMOS

Dissertação apresentada ao Programa de Pós-Graduação em Medicina Veterinária da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do título de **Mestre em Medicina Veterinária.**

Orientadora: Profª Drª. Sônia de Avila Botton
Co-orientadora: Drª. Clarissa Silveira Luiz Vaz

Santa Maria, RS
2017

Daiane Voss Rech

IMPACTO DE TRATAMENTOS DE CAMA AVIÁRIA REUTILIZADA NA VIABILIDADE E INFECTIVIDADE DE MICRO-ORGANISMOS

Dissertação apresentada ao Programa de Pós-Graduação em Medicina Veterinária da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do título de **Mestre em Medicina Veterinária.**

Aprovado em 21 de fevereiro de 2017:

Sônia de Avila Botton, Drª. (UFSM)
(Presidente/Orientadora)

Clarissa S. L. Vaz, Drª. (Embrapa)
(Co-orientadora)

Maristela Lovato, Drª. (UFSM)

Daniela Isabel Brayer Pereira, Drª. (UFPel)

Santa Maria, RS
2017

DEDICATÓRIA

Ao meu amado esposo,

Aos meus amados pais e irmã...

AGRADECIMENTOS

Minha gratidão a todos que, direta ou indiretamente, contribuíram nessa conquista!

À UFSM, agradeço pela oportunidade e em especial:
À professora Sônia pela disponibilidade, incentivo e dedicação;
Aos demais professores do PPGMV e a professora Luciana Potter pelo aprendizado;
Aos colegas de curso, à Bel, Vanessinha, Carla, Tati e Dani pela receptividade e companhia;

À Embrapa, por tudo que me possibilitou alcançar e em especial:
À Clarissa pelo comprometimento, dedicação e apoio. Minha gratidão e admiração!
À Iara, Vica, Liana, Arlei e Cla pelo auxílio e incentivo desde o início.
Ao Dirceu Bassi, sempre gentil, pelo apoio e orientação em todas as etapas;
As chefias pelo incentivo e apoio;
A todos que nos auxiliaram no planejamento e execução do projeto, não teria sido possível sem vocês!
Aos amigos do Laboratório de Sanidade da Embrapa Suínos e Aves, pelo auxílio, alegria, carinho e apoio. Senti saudades!

E aos que estão comigo, onde quer que eu vá:
Ao Beto, meu companheiro de vida, por compreender minha ausência, pelo carinho, compreensão e amor incondicionais. Sou privilegiada em tê-lo em minha vida!
A minha irmã, por me fazer rir, pelo amor e pelo incentivo em tempo integral;
Aos meus pais, sogros e demais familiares, pelo aprendizado mais importante! Por todo carinho e compreensão;
À Raquel e a Tânia pela amizade, carinho, conforto e auxílio. Vocês fazem a diferença! Toda a minha gratidão!
Às Famílias Klein, Rebelatto Ramme, Ubiali e Gaio pelo carinho. Jamais esquecerei!
A Antônia pelo cuidado conosco e nosso lar na minha ausência;
E aos anjos que me guardam pelo privilégio e aprendizado de chegar até aqui, na tentativa de ser um ser humano melhor!

Muito obrigada!

“O tempo tem uma forma maravilhosa de nos mostrar o que realmente importa”

(Caio Fernando Abreu)

RESUMO

IMPACTO DE TRATAMENTOS DE CAMA AVIÁRIA REUTILIZADA NA VIABILIDADE E INFECTIVIDADE DE MICRO-ORGANISMOS

AUTORA: Daiane Voss Rech
ORIENTADORA: Sônia de Avila Botton
CO-ORIENTADORA: Clarissa Silveira Luiz Vaz

A reutilização da cama aviária é uma prática comum na avicultura de corte. Porém, o reuso da cama requer a adoção de procedimentos eficientes na inativação e controle de micro-organismos indesejáveis no intervalo entre os lotes, para preservar a saúde avícola e a qualidade do alimento produzido. A preocupação com as questões sanitárias na avicultura engloba a saúde dos frangos e do consumidor. A produção está sujeita às emergências sanitárias e deve estar preparada para empregar medidas adequadas de contenção e controle. Além disso, os mercados consumidores internacionais demandam comprovação da eficiência dos métodos de tratamento para o reuso da cama entre lotes de frangos. A eficiência dos tratamentos de cama sobre os patógenos é variável e multifatorial e pode ser influenciada pelo método de tratamento e/ou pelos micro-organismos avaliados. Deste modo, o objetivo desta pesquisa foi avaliar a eficiência de diferentes estratégias de tratamento da cama aviária sobre vírus da Doença de Newcastle (VDNC), vírus da Doença Infecciosa da Bursa (VDIB) e *Salmonella* Heidelberg. Enterobactérias totais foram analisadas como indicador da qualidade microbiológica da cama aviária. Inicialmente, a contaminação experimental pelos vírus aviários foi padronizada, comparando-se a excreção por aves inoculadas (*seeder birds*) *versus* a aspersão direta dos vírus na cama. Para avaliação dos tratamentos, cama aviária reutilizada foi contaminada com os três micro-organismos e submetida aos tratamentos (T): T1- fermentação plana; T2- cal virgem; T3- fermentação plana seguida de adição de cal virgem; T4- não tratado. A cama aviária foi submetida às análises bacteriológicas e físico-químicas durante o tratamento. Aves sentinelas foram alojadas sobre a cama tratada, sendo monitoradas por meio de avaliação clínica e análises microbiológicas, sorológicas e moleculares. Os resultados demonstraram que as *seeder birds* foram eficientes em estabelecer a contaminação viral da cama. O T1 foi superior na redução de enterobactérias totais na cama aviária. A avaliação das aves sentinelas indicou que ambos T1 e T3 inativaram VDIB na cama aviária. O T2 não foi eficiente sobre os micro-organismos avaliados e sua associação ao T1 (T3) não potencializou a ação do tratamento. O VDNC não sobreviveu na cama, independente do tratamento aplicado. *S.* Heidelberg permaneceu viável na cama de todos os tratamentos, sendo também detectada nas aves sentinelas. A atividade antimicrobiana dos T1 e T3 foi relacionada aos maiores teores de amônia presentes na cama aviária. Os resultados indicam que a fermentação plana é eficiente para o controle do VDIB e enterobactérias totais residuais na cama aviária reutilizada. Todavia, na presença de *S.* Heidelberg outras alternativas devem ser consideradas no controle deste agente de importância na saúde animal e pública.

Palavras-chave: Cama de aviário. Tratamento. Frango de corte. Vírus da Doença de Newcastle. Vírus da Doença Infecciosa da Bursa. *Salmonella* Heidelberg.

ABSTRACT

IMPACT OF TREATMENTS FOR RECYCLED BROILER LITTER ON VIABILITY AND INFECTIVITY OF MICROORGANISMS

AUTHOR: Daiane Voss Rech
ADVISOR: Sônia de Ávila Botton
CO-ADVISOR: Clarissa Silveira Luiz Vaz

Reusing litter is a common practice in broiler farming. However, it requires the adoption of efficient procedures for inactivating and controlling residual microorganisms during downtime between flocks to ensure sanitary control over the next flock and the quality of the broiler meat. The broiler production adopts a series of stringent precautionary measures to avoid sanitary emergencies and should be able to employ appropriate control measures. In addition, international consumer markets require proof of the efficiency of treatment methods for broiler litter reuse. The efficiency of broiler litter treatments on pathogens is variable and multifactorial and can be influenced by the treatment method and/or microorganisms evaluated. This study aimed to evaluate the efficiency of different strategies of treatment of broiler litter on Newcastle disease virus (NDV), Infectious bursal disease virus (IBDV) and *Salmonella* Heidelberg. Total enterobacteria counts were carried out as an indicator of microbiological quality of litter. First, the experimental contamination of the broiler litter by viruses was standardized, comparing the seeder birds inoculated versus the direct spray of the virus in the broiler litter. To evaluate the treatments, reused broiler litter was contaminated with the three microorganisms and submitted to the treatments (T): T1- shallow fermentation; T2- quicklime; T3- shallow fermentation followed by quicklime; and, T4- untreated. The broiler litter was submitted to bacteriological and physico-chemical analyzes during treatment. Sentinel chicks were housed on the broiler litter treated and further monitored by clinical evaluation, as well as microbiological, serological and molecular tests. The results demonstrated that seeder birds were efficient to stablish viral contamination in broiler litter. T1 was superior in reducing total enterobacteria in the broiler litter. The evaluation of sentinel chicks also indicated that T1 and T3 inactivated IBDV in the broiler litter. T2 was not able to reduce the microorganisms evaluated, and its association with T1 (T3) did not enhance the treatment action. NDV did not survive in broiler litter, regardless of the treatment applied. *S*. Heidelberg survived in broiler litter after all treatments evaluated and was also detected in the sentinel chicks. The antimicrobial activity of T1 and T3 was associated to ammonia levels present in the broiler litter. The results reveal that shallow fermentation is efficient to control residual IBDV and total enterobacteria in recycled broiler litter. However, other strategies should be considered in the presence of S. Heidelberg.

Keywords: Broiler litter. Treatment. Broiler. Newcastle Disease Virus. Infectious Bursal Disease Virus. *Salmonella* Heidelberg.

LISTA DE ABREVIATURAS E SIGLAS

AIC	*Akaike Information Criterion*
APHA	*American Public Health Association*
BGA	*Brilliant Green Agar*
CaO	Óxido de cálcio
CDC	*Centers for Disease Control and Prevention*
CFU	*Colony Forming Unit*
COBEA	Colégio Brasileiro de Experimentação Animal
cm	Centímetros
Cq	*Quantification Cycle*
DMSO	*Dimethyl sulfoxide*
DNC	Doença de Newcastle
EFSA	*European Food Safety Authority*
EID_{50}	*Fifty percent Embryo Infectious Dose*
ELISA	*Enzyme-Linked Immunosorbent Assay*
EUA	Estados Unidos da América
GLOBALG.A.P.	Sistema de Certificação Global em Boas Práticas Agrícolas
IA	Influenza Aviária
IAL	Instituto Adolfo Lutz
IBDV	*Infectious Bursal Disease Virus*
IN	Instrução Normativa
IU	*International Units*
L	*Liter*
m^2	Metro quadrado
MAPA	Ministério da Agricultura Pecuária e Abastecimento
mL	*Mililiter*
NDV	*Newcastle Disease Virus*
NH_3	Amonia
$N\text{-}NH_4^+$	Nitrogênio amoniacal
OIE	Organização Mundial de Saúde Animal
PBS	*Phosphate Buffered Saline*
PCR	*Polymerase Chain Reaction*
pH	Potencial de Hidrogênio
RASFF	*Rapid Alert System for Food and Feed*
RNA	*Ribonucleic acid*
RT-qPCR	*Reverse Transcription and Quantitative Real Time Polymerase Chain Reaction*
SPF	*Specific Pathogen Free*
TPB	*Tryptone Peptone Broth*
UFC	Unidades Formadoras de Colônias
µg	Micrograma
USA	*United States of America*
USDA	*United States Department of Agriculture*
VDIB	Vírus da Doença Infecciosa da Bursa
VDNC	Vírus da Doença de Newcastle
VIA	Vírus da Influenza Aviária
XLT4	Ágar Xilose Lisina Tergitol 4

SUMÁRIO

1 INTRODUÇÃO

A avicultura de corte é um dos segmentos produtivos mais significativos para o Brasil. O País é o segundo maior produtor e líder na exportação de carne de frango. No mercado interno, representa uma fonte proteica bastante acessível e muito consumida pelos brasileiros. Fatores como qualidade, sanidade e preço contribuíram para incrementar a produtividade (MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO, 2014). Entretanto, a Organização das Nações Unidas prevê que a população mundial será superior a nove bilhões de pessoas até a metade desse século e que será necessário dobrar a produção mundial de alimentos até 2050. Este aumento da produtividade depende da habilidade de intensificar a atividade agropecuária, garantindo o fornecimento de alimentos seguros, nutritivos, de custo acessível e produzidos de forma sustentável. Estimativas indicam que a taxa de crescimento anual da produção da carne de frango deve alcançar 4,22% (MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO, 2014). O aumento na produção elevará a demanda por insumos, bem como, o volume de resíduos gerados, salientando a importância da gestão sustentável da produção.

A cama aviária é um importante insumo para a avicultura. Em contrapartida, também representa um dos principais resíduos da produção de frangos, além de contribuir com uma fração significativa do custo da produção (MARCOLIN, 2006). Sendo assim, a reutilização da cama aviária entre lotes de frangos vem sendo empregada em vários países, incluindo o Brasil. Entretanto, a cama recebe todas as excretas das aves que são acumuladas ao longo dos lotes e pode ser fonte de propagação de patógenos se não for adequadamente tratada. Portanto, devido ao risco sanitário e suas implicações na produção e saúde pública, é importante que a cama reutilizada seja submetida a um tratamento eficiente no período de vazio entre lotes para reduzir o risco microbiológico (MACKLIN et al., 2006; ÁVILA et al., 2007; GLOBALG.A.P., 2016). Adicionalmente, a Instrução Normativa (IN) 10/2013 do MAPA, que regulamenta a gestão de risco diferenciado em estabelecimentos avícolas, determina que a cama de núcleos positivos para *Salmonella* spp. deve ser submetida a tratamento capaz de inativar a bactéria (BRASIL, 2013). Da mesma forma, a IN 17/2006 do MAPA, que estabelece o Plano Nacional de Controle e Prevenção da Influenza Aviária (IA) e da Doença de Newcastle (DNC) (BRASIL,

2006), autoriza o trânsito interestadual de cama de aviário somente após a inativação de patógenos. Mais recentemente, a IN 21/2014, que define a compartimentação da produção avícola quanto ao *status* sanitário para os vírus da IA e DNC, reforça que a cama reutilizada precisa ser submetida a tratamento com eficácia cientificamente comprovada para inativar ambos os patógenos (BRASIL, 2014). Igualmente, os processos de certificação para exportações à Comunidade Europeia exigem que os riscos microbiológicos da reutilização da cama sejam avaliados rotineiramente (GLOBALG.A.P., 2016). Em todos esses casos, as normativas não especificam quais métodos e/ou tempos de tratamento devem ser aplicados à cama. Desta forma, é importante que estudos científicos sejam desenvolvidos para avaliar a ação dos tratamentos de cama sobre patógenos relevantes para a produção avícola, respaldando as práticas adotadas.

Diferentes métodos de tratamento da cama de aviário vêm sendo utilizados com o objetivo de reduzir o risco microbiológico. No Brasil, os métodos mais comuns são a fermentação em leira, a fermentação plana e a aplicação de cal (ROSA et al., 2014). O método de fermentação em leira é internacionalmente utilizado com conhecida atuação sobre patógenos (MACKLIN et al., 2006; SILVA et al., 2007; CRESPO et al., 2016). O uso de cal também é uma prática comum no Brasil, mas os estudos têm apresentado resultados contraditórios (BENNETT et al., 2005; SILVA et al., 2007; SILVA et al., 2009; LOPES et al., 2015). Por outro lado, a fermentação plana da cama é uma metodologia mais recente, desenvolvida no Brasil, com bons resultados no controle de enterobactérias e *Salmonella* Enteritidis (SILVA et al., 2007; SILVA et al., 2009). Todavia, são escassos os estudos envolvendo outros patógenos (LOPES et al., 2015). Desta forma, considerando que diferentes micro-organismos podem apresentar grandes variações na suscetibilidade às condições ambientais (ISLAM et al., 2013), é importante que os tratamentos sejam avaliados sobre os mais diversificados patógenos. Neste contexto, destacam-se *Salmonella* Heidelberg e os vírus aviários com impacto à defesa sanitária.

S. Heidelberg tem causado preocupação às empresas avícolas brasileiras, devido a sua resistência no ambiente (informação verbal)[1] e está entre os sorovares mais prevalentes nos EUA (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2015; UNITED STATES DEPARTMENT OF AGRICULTURE, 2016).

[1] Palestra apresentada por Dr. Alberto Back no XVI Simpósio Brasil Sul de Avicultura, Chapecó, SC, abril de 2015.

A identificação de *S.* Heidelberg multirresistente aos antimicrobianos (ROTHROCK Jr et al., 2015) e a ocorrência de surtos interestaduais de infecção humana ligada ao consumo de carne de frango nos EUA (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2013) denotam sua importância para a segurança dos alimentos. *S.* Heidelberg não está entre os sorovares mais frequentemente detectados em carne de frango produzida na Comunidade Europeia (EUROPEAN FOOD SAFETY AUTHORITY, 2015). Contudo, as notificações emitidas pelo sistema europeu de alerta rápido para alimentos quanto à presença de salmonelas na carne de frango brasileira, incluindo *S*. Heidelberg, são mais numerosas em relação aos outros perigos monitorados e tem sido restritiva a ponto de impedir a entrada do produto na Europa (RAPID ALERT SYSTEM FOR FOOD AND FEED, 2015). Trata-se, portanto, de uma barreira ao comércio internacional da carne de frango brasileira e reforça a necessidade de intervenções para reduzir a prevalência da bactéria nos aviários.

A DNC e a IA são altamente contagiosas e fazem parte da lista de doenças emergenciais do código zoossanitário internacional da Organização Mundial de Saúde Animal (OIE). A ocorrência dessas doenças no plantel avícola brasileiro acarretaria danos econômicos inestimáveis, incluindo o impedimento da comercialização, o cancelamento das exportações, o sacrifício e a quarentena das aves, bem como um longo trabalho para reestabelecer a condição sanitária (BRASIL, 2006). Portanto, a prevenção e controle desses vírus são fundamentais. O vírus da Doença Infecciosa da Bursa (VDIB) é endêmico na avicultura e a doença é prevenida por meio da vacinação regular dos frangos (BERNARDINO e LEFFER, 2009), entretanto surtos eventuais continuam sendo relatados (MÜLLER et al., 2003; CRESPO et al., 2016). Além disso, o VDIB tem sido considerado indicador da permanência de outros vírus menos sensíveis na cama aviária, incluindo o vírus da Doença de Newcastle (VDNC) e o vírus da Influenza Aviária (VIA) (GUAN et al., 2010). Por razões de biosseguridade, a experimentação com cepas virulentas da DNC e IA é restrita, mas é possível estabelecer relações biológicas utilizando um modelo viral vacinal.

Nesse cenário, este trabalho foi desenvolvido com o objetivo de avaliar o efeito de diferentes tratamentos sobre *Salmonella* Heidelberg, VDIB e VDN na cama aviária reutilizada, permitindo identificar procedimentos a serem recomendados em episódios sanitários ou como método de rotina quando da reutilização da cama entre lotes.

2 REVISÃO BIBLIOGRÁFICA

2.1 CAMA DE AVIÁRIO: A MICROBIOTA E OS PATÓGENOS

A cama aviária tem o papel de absorver a umidade das excretas e do ambiente; diluir as excretas, diminuindo o contato com as aves; atuar como isolante térmico, impedindo o contato da ave direto com o piso e reduzindo as oscilações de temperatura no galpão; fornecer conforto à ave, proporcionando uma superfície macia (ÁVILA et al., 2007; RITZ, et al., 2014), bem como, permite a expressão do instinto natural de ciscar e banhar-se das aves (BRACKE e HOPSTER, 2006).

A deposição de micro-organismos a partir das excretas dos frangos desempenha um papel importante na formação da microbiota da cama do aviário. Ao final do lote, a cama apresenta micro-organismos da excreta das aves, além daqueles do próprio substrato ou carreados pelo ar, pessoas, vetores e equipamentos, atingindo concentrações próximas a 1 bilhão por grama (LU et al., 2003). A composição e variedade desta microbiota dependem do número de aves alojadas, e podem sofrer modificações de acordo com a idade e dieta das aves (WADUD et al., 2012). Abordagens moleculares têm sido empregadas para caracterizar a microbiota da cama de aviário, permitindo identificar os gêneros bacterianos predominantes, tais como *Lactobacillus, Pediococcus, Salinicoccus, Facklamia, Staphylococcus, Corynebacterium, Jeotgalicoccus*,*Virgibacillus, Brevibacterium* e *Bacillus* (LU et al., 2003; CRESSMAN et al., 2010; WADUD et al., 2012).

Embora numericamente menos expressivos, importantes patógenos aviários estão frequentemente presentes na cama de frangos de corte (DAVIES e WRAY, 1996; LU et al., 2003; FIORENTIN, 2006; ROLL; DAI PRÁ; ROLL, 2011; CRESPO et al., 2016). Entre esses micro-organismos destacam-se *Salmonella* spp. e *Campylobater* termófilos, implicados em problemas inerentes à segurança dos alimentos, bem como *Escherichia coli*, especialmente as linhagens patogênicas para aves (APEC) e causadoras de dermatite necrótica nos frangos. Outras bactérias, como *Clostridium perfringens* e *Staphylococcus aureus* também podem eventualmente causar infecções oportunistas e/ou levar à contaminação das carcaças no processamento (SILVA, 2011).

Vírus aviários, especialmente os que apresentam replicação entérica, também podem ser perpetuados na cama aviária (MÜLLER et al., 2003; GUAN et al., 2010; ISLAM et al., 2013; CRESPO et al., 2016). Destes, destacam-se o VIA e o VDNC, por sua importância para a defesa sanitária avícola como doenças emergenciais. Além destes, o VDIB, que é muito infeccioso e resistente à inativação (MÜLLER et al., 2003; CRESPO et al., 2016), tem sido recomendado como modelo para predizer a inativação de outros vírus menos resistentes na cama aviária, incluindo poxvirus, adenovirus, reovirus, VDNC e VIA (GUAN et al., 2010).

Salmonella spp. continuam sendo os agentes bacterianos de maior preocupação para a avicultura e causam uma das doenças transmitidas por alimentos mais prevalentes em todo o mundo (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2015; EUROPEAN FOOD SAFETY AUTHORITY, 2015). Existem mais de 2500 sorovares com ampla diversidade de reservatórios animais e capacidade de sobrevivência em ambientes adversos. Há dois grupos de *Salmonella* spp. que são de interesse para a avicultura: as salmonelas tíficas (*S.* Pullorum e *S*. Gallinarum), que são específicas das aves e nas quais causam doença clínica com perdas produtivas e econômicas impactantes; e as salmonelas paratíficas, que incluem os sorovares não restritos a um hospedeiro específico, como *S.* Heidelberg, que normalmente cursam com infecção subclínica em aves adultas, porém são potencialmente capazes de causar a contaminação dos alimentos e infecção humana (SHIVAPRASAD et al., 2013).

A presença de *Salmonella* spp. na cama de aviário é crítica para a permanência da bactéria nas granjas, cuja fonte de contaminação pode ser os próprios pintos, vetores e porções de cama não submetidas ao tratamento entre lotes (DAVIES e WRAY, 1996). *Salmonella spp.* podem sobreviver no ambiente por longos períodos de tempo, chegando a 18 meses na cama aviária (WILLIAMS e BENSON, 1978). Algumas cepas podem sobreviver em extremos de temperatura (2°C a 54°C) e em ambientes com baixa umidade (ANDINO e HANNING 2015). A produção de biofilme e mecanismos adaptativos de tolerância aos fatores de stress também contribuem para a persistência de *Salmonella* spp. no ambiente. (ANDINO e HANNING 2015). A dificuldade de controle facilita sua perpetuação na produção avícola e a possibilidade de contaminação das carcaças no abate, com as devidas penalidades dentro do Programa de Redução de Patógenos do MAPA (BRASIL, 2003).

Dentre as doenças virais, a DNC é uma enfermidade viral aguda, altamente contagiosa, com sinais respiratórios, manifestações nervosas, diarreia e edema da cabeça. Faz parte da lista de doenças emergenciais da OIE e a notificação dos focos da doença é compulsória quando causada por vírus, cuja patogenicidade intracerebral em pintos SPF de um dia, seja maior que 0,7 (WORLD ORGANIZATION FOR ANIMAL HEARTH, 2016). O VDNC pode persistir no ambiente por mais de uma semana, tempo suficiente para ser perpetuado ao lote de frangos subsequente, se nenhum método de controle eficiente for praticado (WORLD ORGANIZATION FOR ANIMAL HEARTH, 2016).

A DIB ou doença de Gumboro é aguda e contagiosa, afeta a bolsa de Fabricius, leva a falta de apetite, diarreia, depressão, desuniformidade, mortalidade e imunossupressão (ENTERADOSSI e SAIF, 2008). A imunossupressão predispõe as aves a uma variedade de infecções secundárias. Adicionalmente, as aves podem apresentar uma reposta imune insatisfatória na vacinação contra outros micro-organismos (MÜLLER et al., 2012). O VDIB não possui envelope, o que lhe confere maior estabilidade ao ambiente. Entretanto, é não-infectivo acima de 42°C e instável acima de 72°C, sendo que mudanças no pH não contribuem para sua estabilidade (RANI e KUMAR, 2015). É capaz de sobreviver em instalações por até 100 dias e a alta resistência a diferentes compostos químicos resulta em longa viabilidade, mesmo quando aplicadas rigorosas medidas de limpeza e desinfecção (ENTERADOSSI e SAIF, 2008).

2.2 REUTILIZAÇÃO DA CAMA DE AVIÁRIO

O alojamento de frangos sobre cama aviária reutilizada promove alteração da microbiota intestinal (CRESSMAN et al., 2010). Contudo, esta alteração não é necessariamente negativa. Estudos prévios demostram que a cama reutilizada entre lotes e adequadamente manejada não é prejudicial às aves; contudo, resulta em lotes com menor frequência de problemas sanitários, menor mortalidade e índices zootécnicos de produtividade similares ou superiores aos observados nos lotes criados em cama nova (JONES e HAGLER, 1983; RITZ; FAIRCHILD; LACY, 2014). Sugere-se que a cama reutilizada também tem efeito inibitório sobre *Salmonella* spp., quando comparada a cama nova (CORRIER; HARGIS; HINTON, 1992; ROLL; DAI PRÁ; ROLL, 2011).

A proteção da colonização intestinal em frangos criados sobre cama reutilizada pode ser atribuída à exclusão competitiva por sítios de adesão e produção de ácidos voláteis que reduzem o pH cecal (ZIPRIN et al., 1991) e estimulam o sistema imunológico aviário (OVIEDO-RONDON, 2009). Bactérias ácido-láticas presentes na cama podem produzir bacteriocinas que contribuem com a saúde intestinal (HIGGINS et al., 2008). Adicionalmente, alguns vírus vacinais sobrevivem na cama reutilizada entre lotes de frangos (ISLAM et al., 2013) e podem estimular a proteção imunológica do lote subsequente.

Em termos econômicos, a substituição da cama de aviário a cada lote de frangos aumenta cerca de 77% o custo da produção avícola (MARCOLIN, 2006). Além disso, a escassez de bons materiais para servir de substrato à cama e o aumento de preço das matérias primas também dificultam a viabilidade da produção nas diversas regiões do País. Na ótica ambiental, o reuso da cama por 6 lotes preserva aproximadamente 95.200 árvores/ano e gera cinco vezes menos volume de resíduo se usada por um único lote (MARCOLIN, 2006).

A IN 08/2004 do MAPA proibiu o uso de produtos de origem animal e resíduos da produção, incluindo cama de aviário, na alimentação de ruminantes como meio preventivo das encefalopatias espongiformes (BRASIL, 2004). Deste modo, o principal destino do resíduo de cama aviária é o uso como fertilizante agrícola. Cada quilograma de ave produzida gera de 1,2 a 1,5 Kg de resíduo de cama (ISLAM et al., 2013). Em regiões de alta concentração da produção avícola, entretanto, o descarte da cama de aviário a cada lote extrapolaria a capacidade de reuso como adubo frente ao excedente nutricional no solo (MARCOLIN, 2006). Porém, do ponto de vista sanitário, a cama, que recebe as excretas das aves ao longo dos lotes, pode ser uma fonte de propagação de patógenos, por isso, seu reuso no aviário de frangos de corte requer procedimentos eficientes na inativação de micro-organismos indesejáveis (ÁVILA et al., 2007).

2.3 TRATAMENTOS DE CAMA AVIÁRIA ENTRE LOTES

Existem vários métodos de manejo de cama voltados à inativação e controle de patógenos entre lotes, bem como há variações em um mesmo tratamento. A rigor, não há exigência para a unificação dos procedimentos, porém alguns mercados têm exigido comprovação científica de sua eficiência (GLOBALG.A.P.,

2016). Formulado na Europa, a partir de 1997, o GlobalG.A.P. é uma organização que atua na definição de padrões de boas práticas agrícolas e é o principal programa certificador de garantia da qualidade agrícola mundial. O GlobalG.A.P., por exemplo, estabelece no documento "*Control points and compliance criteria*" (ítem 5.5.5): "A cama reutilizada deve ser tratada e comprovadamente livre de riscos microbiológicos" (GLOBALG.A.P., 2016).

No Brasil, os métodos de tratamento mais frequentemente utilizados são a fermentação em leira, a aplicação de cal e a fermentação plana (ROSA et al., 2014). Os tratamentos de cama aviária são aplicados após a despopulação, concomitante com a lavagem e desinfecção dos equipamentos. Considerando-se as variações de cada integração, os tratamentos baseiam-se nas etapas descritas a seguir, adaptadas de Rosa et al. (2014):

2.3.1 Fermentação em leira

a. Queima de penas com lança-chamas;
b. Remoção de cama úmida e crostas do aviário;
c. Amontoamento da cama, fazendo uma pilha ou leira no centro do galpão, ao longo do aviário;
d. Cobertura da leira com lona plástica por 10-12 dias (período de fermentação);
e. Remoção da lona e redistribuição da cama tratada no aviário, exceto na área dos pinteiros (25%);
f. Queima de penas residuais com lança-chamas;
g. Ventilação do aviário por 2 a 3 dias antes do alojamento;
h. Adição de cama nova na área destinada ao pinteiro.

2.3.2 Fermentação plana

a. Umedecimento da cama utilizando cerca de 20 litros de água por metro linear;
b. Revestimento da base (1 m) dos pilares centrais do aviário com lona;
c. Remoção da cama das paredes laterais do aviário abrindo um sulco entre as paredes e a cama, para colocação da lona;

d. Cobertura da cama com lona em toda a extensão do aviário, colocando as laterais e extremidades da lona rente ao piso, por baixo da camada de cama, para evitar a entrada de ar;
e. Remoção da lona após 10 dias de fermentação, retirando as crostas e revolvendo a cama em todo o aviário;
f. Queima de penas com lança-chamas;
g. Ventilação do aviário por 2 dias antes do alojamento;
h. Adição de cama nova na área destinada ao pinteiro.

2.3.3 Aplicação de cal

a. Remoção de cama úmida e crostas do aviário;
b. Queima de penas com lança-chamas;
c. Distribuição de CaO (cal virgem) em todo o galpão (mínimo de 3,6 Kg/m^3), até 72 horas antes do alojamento, incorporando uniformemente o produto na cama;
d. Queima de penas com lança-chamas;
e. Adição de cama nova na área destinada ao pinteiro.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar a viabilidade e infectividade do vírus da Doença de Newcastle, vírus da Doença Infecciosa da Bursa e *Salmonella* Heidelberg em cama aviária reutilizada, submetida a diferentes tratamentos.

3.2 OBJETIVOS ESPECÍFICOS

- Padronizar o protocolo de contaminação experimental da cama aviária com o vírus da Doença de Newcastle e o vírus da Doença Infecciosa da Bursa;
- Otimizar a técnica de Reação em cadeia da polimerase em tempo real por transcriptase reversa (RT-qPCR) para detecção do vírus da Doença Infecciosa da Bursa;
- Avaliar o efeito dos diferentes tratamentos sobre *Salmonella* Heidelberg e enterobactérias totais na cama de aviário reutilizada no período de tratamento da cama;
- Determinar os níveis de temperatura, material seca, pH e amônia na cama aviária submetida aos diferentes tratamentos;
- Determinar o efeito da contaminação residual dos vírus da Doença de Newcastle, vírus da Doença Infecciosa da Bursa e *Salmonella* Heidelberg na cama de aviário tratada usando um modelo biológico (aves);

4 MANUSCRITO – IMPACT OF TREATMENTS FOR RECYCLED BROILER LITTER ON VIABILITY AND INFECTIVITY OF MICROORGANISMS

(Encaminhado para publicação à revista Veterinary Microbiology, ISSN 0378-1135)

Impact of treatments for recycled broiler litter on the viability and infectivity of microorganisms

Daiane Voss-Rech[a,c]*, Iara Maria Trevisol[a], Liana Brentano[a], Virgínia Santiago Silva[a], Raquel Rebelatto[a], Fátima Regina Ferreira Jaenisch[a], Cintia Hiromi Okino[b], Marcos Antonio Zanella Mores[a], Arlei Coldebella[a], Sônia de Avila Botton[c#], Clarissa Silveira Luiz Vaz[a]

[a]Embrapa Suínos e Aves, BR 153, Km 110, CEP: 89715-899, Concordia, SC, Brazil.

[b]Embrapa Pecuária Sudeste, Rodovia Washington Luiz, Km 234, CEP: 13560-970, São Carlos, SP, Brazil.

[c]Programa de Pós-graduação em Medicina Veterinária (PPGMV). Departamento de Medicina Veterinária Preventiva, Centro de Ciências Rurais (CCR), Universidade Federal de Santa Maria (UFSM), Avenida Roraima, 1000, CEP: 97105-900, Santa Maria, RS, Brazil. [#]Adviser PPGMV/CCR/UFSM

*Corresponding Author

Phone: +55 (49) 3441-3278, fax: +55 (49) 3441-0497, e-mail: daiane.rech@embrapa.br

Abstract

The microbiological risk of recycled litter depends on the efficacy of the management system applied to inactivate residual microorganisms and preserve the health of the successive broiler flock. This study aimed to assess the viability and infectivity of the Newcastle Disease Virus (NDV), Infectious Bursal Disease Virus (IBDV) and *Salmonella* Heidelberg in recycled litter exposed to different treatments. The litter was contaminated with microorganisms and submitted to the treatments (T): T1: shallow fermentation; T2: quicklime (calcium oxide); T3: shallow fermentation followed by addition of quicklime; T4: no treatment. Sentinel chicks housed on the treated litter showed that T1 and T3 inactivated residual IBDV. Analysis of the litter subjected to T1 also showed reduced levels of total enterobacteria. T2 was not able to reduce the microorganisms assessed and its association with T1 (T3) failed to enhance the effect of the treatment. NDV did not survive in the broiler litter, regardless of the treatment applied, and it was also not detected in the sentinel chicks. *S.* Heidelberg remained viable in the litter submitted to all studied treatments, being isolated from the sentinel chicks of all the experimental groups. The antimicrobial activity of T1 and T3 was associated to higher ammonia contents in the broiler litter. The results indicate that the shallow fermentation treatment is efficient for controlling residual IBDV and total enterobacteria in the recycled litter.

Key words: litter treatment; broiler; Newcastle Disease Virus; Infectious Bursal Disease Virus; *Salmonella* Heidelberg.

1. Introduction

Broiler industry in Brazil operates with an integrated production model between farmers and processing companies. The broilers are raised in broiler houses on litter, usually composed of wood shavings and sawdust, but the substrate varies according to the region and availability (Torok et al., 2009). Multiple broiler flocks are raised in the all in/all out system on a single litter batch, which is recycled according to the instructions of the processing company. During the downtime between flocks, caked portions are removed, residual feathers are burned with a flame thrower and the litter is treated in order to reduce the microbial load for receiving the next broiler flock (Macklin et al., 2006).

Viable microorganisms remaining in the recycled litter interferes with the broilers' health, and can have a detrimental effect on productivity, trade restrictions or the safety of the broiler meat. Non-typhoidal *Salmonella* serovars are relevant concerns for poultry production as they are potential food contaminants (Shivaprasad et al., 2013). In Brazil, the prevalence of *S.* Heidelberg has been increasing in broiler production (Pulido-Landínez et al., 2013; Voss-Rech et al., 2015) and there is still a lack in the understanding about the role of litter management on the bacteria elimination at the end of the production cycle at farm level.

Broiler litter is also a potential source of avian viruses, especially those that are shed in stools, such as the Newcastle Disease Virus (NDV) and the Infectious Bursal Disease Virus (IBDV) (Müller et al., 2003; Guan et al., 2009; Guan et al., 2010; Islam et al., 2013; Crespo et al., 2016). Residual viruses may originate from infections with or without apparent clinical signs of illness, and also from the prophylactic use of live vaccines. On the other hand, live vaccine strains are susceptible to mutations and genomic variation that can result in the emergence of antigenic variant strains

(Fleischmann, 1996). Moreover, failures in prophylactic management and/or in biosecurity programs (Müller et al., 2012) favor the eventual spread of the disease within a flock. Thus, efficient farming practices, which include litter treatment, are essential to prevent persistence of pathogens in the broiler house. For reasons of biosecurity, experimenting with pathogenic strains of NDV and IBDV is highly restricted, but it is possible to establish biological relations using a viral vaccine model. IBDV is highly infectious and very resistant to inactivation (Müller et al., 2003; Crespo et al., 2016). Overall, it has been recommended as a model to predict the inactivation of less resistant viruses during litter treatment, such as poxvirus, adenovirus, NDV, reovirus and avian influenza virus (Guan et al., 2010).

In Brazil, the most commonly used methods to reduce microorganisms in litter recycled between broiler flocks are windrowing, shallow fermentation and the addition of quicklime (Vaz et al., 2017; Lopes et al., 2015). In a previous study, we demonstrated that windrowed litter produced a higher reduction of aerobic mesophiles, whereas shallow fermentation resulted in lower levels of total enterobacteria and all treatments reduced *S.* Enteritidis in recycled litter (Vaz et al., 2017). Windrowing is used in several countries with good results (Macklin et al., 2006; Vaz et al., 2017; Chen et al., 2015; Crespo et al., 2016). On the other hand, studies that have assessed the addition of quicklime have reported contradictory results (Bennett et al., 2005; Ruiz et al., 2008; Vaz et al., 2017; Lopes et al., 2015). Shallow fermentation, a more recent and widely applied method in Brazil, has not yet been more thoroughly studied (Vaz et al., 2017; Lopes et al., 2015) and its effect on the inactivation and/or reduction in the spread of avian viruses remains unknown.

The infectivity of the residual microorganisms is a critical factor for evaluating the efficacy of treatments to reduce the microbial load in recycled litter. Some

microorganisms may not be detected by methods that require viable or high microbial titer, due to environmental degradation and/or very high contamination levels of the litter. Nevertheless, these microorganisms can multiply in more favorable conditions, such as by the infection of a new not immune live host (Islam et al., 2013; Crespo et al., 2016). In this context, this study aimed to evaluate the viability and the infectivity of NDV as a model for very liable enveloped viruses, IBDV as a non-enveloped virus very resistant to inactivation and *S.* Heidelberg due to its increased prevalence in broiler chickens, in recycled broiler litter submitted to different treatments.

2. Materials and Methods

2.1 Animal ethics

All the chicks used in this study were treated in accordance to the standards of animal welfare and ethics adopted by the Brazilian Board for Animal Experimentation (*Colégio Brasileiro de Experimentação Animal*, COBEA), and the experimental animal protocol approved by the Embrapa Swine and Poultry Ethics Committee for Animal Experimentation (no. 003/2015).

2.2 Standardization of the viral contamination of the litter

Two methods were evaluated for experimentally controlled litter contamination with NDV and IBDV vaccine strains: (1) via live virus vaccine inoculated seeder birds and (2) via direct virus vaccine spraying. The litter used was of recycled wood shavings and sawdust from a commercial broiler producer and previously tested as negative for both viruses. The litter was distributed in two concrete-floored experimental boxes measuring 4 m^2 in enough quantity to form a 10 cm high litter

layer. By the seeder birds method, 48 White Leghorn specific pathogen free (SPF) chicks at 10 days of age (12 birds/m^2) were inoculated orally with $10^{3.1}$ 50% embryo infectious dose (EID_{50}) of IBDV (vaccine strain GBV8) and intranasally with $10^{5.6}$ EID_{50} of NDV (vaccine strain La Sota), and housed on the litter for 7 days. On days 3, 5 and 7 after inoculation (pi) litter samples were collected for reverse transcription and quantitative real time polymerase chain reaction (RT-qPCR) and viral isolation; and cloacal and oropharyngeal swabs were collected from the chicks and tested by NDV and IBDV RT-qPCR, in order to define the ideal time to obtain maximum viral shedding. In the direct spraying method, the litter was left to settle for seven days and then the vaccine strains of IBDV ($10^{5.7}$ EID_{50}) and NDV ($10^{8.2}$ EID_{50}) were sprayed over the litter. On days 3 and 5 after contamination, litter samples were taken to test for IBDV and NDV by RT-qPCR and viral isolation. After removal of the seeder birds or after the direct spraying, the litter was left to settle, without any intervention, for five days. On the sixth day, 10 SPF sentinel chicks at 10 days of age were housed on the contaminated litter in each box to evaluate the presence of residual vaccine viruses. On the fifth day of housing, the birds were euthanized and swabs from the cloaca and trachea were collected to test for IBDV and NDV by RT-qPCR.

2.3 Experimental design

Five experimental groups were formed to evaluate the following treatments (T) in the recycled litter: T1 - shallow fermentation; T2 - quicklime (calcium oxide); T3 - shallow fermentation followed by addition of quicklime; T4 - no treatment. Additionally, T5 consisted of a fresh wood shavings litter, not contaminated and untreated, and had the objective of monitoring the absence of cross contamination

between the other treatments. The treatments were conducted in experimental facilities, in individual rooms, with four repetitions in 2 m^2 boxes for each treatment. Each room had an antechamber and footbath and different teams had access to each separate treatment. For each entry, clean clothes were worn and gloves and shoe covers were replaced between boxes.

2.4 Contamination of the litter

Recycled litter, previously used to raise six broiler flocks, was obtained from a commercial broiler producer, immediately after the removal of the broilers and tested negative for *Salmonella* spp., IBDV and NDV. The broiler litter was transported to the experimental facilities and distributed in the boxes in sufficient volume to form a 10 cm high bedding. Viral contamination was conducted by the seeder bird method, as described (2.1), modifying the NDV dose to $10^{6.6}$ EID_{50}. On the sixth day of housing, the seeder birds were removed and oropharyngeal and cloacal swabs were collected to confirm viral replication by RT-qPCR for NDV and IBDV, respectively. Next, the litter was sprayed with a field strain of *S.* Heidelberg resistant to streptomycin and gentamicin (1.6 x 10^9 CFU/m^2) and then turned, as previously established (Vaz et al., 2017).

2.5 Litter treatment

The treatments were initiated immediately after contamination of the broiler litter (day 0) for a 14-day period. In T1 and T3 the litter was moistened with 1.5 L/m^2 of distilled water and covered with a 200 µm non-breathable tarpaulin laid out so that the edges were tucked in under the layer of litter to prevent gas exchanges with the

external environment. On day 12, the tarpaulin was removed in both treatments, with 600 g/m^2 of quicklime being incorporated into the litter in T3, corresponding to approximately 2% of the litter mass. The T1 and T3 litters were left to settle for two days. In T2 the litter was left to settle after contamination and on the 12th day 600 g/m^2 of quicklime was added. In T4 and T5 the litter was left in repose for 14 days. Litter samples of each treatment was collected in pools at five points in each box on days 0, 6, 12 and 14 for quantification of total enterobacteria, isolation and quantification of *S.* Heidelberg, pH, dry matter and ammonia content determination. In T1 and T3, the broiler samples contained in 20 x 10 cm polyester mash bags were distributed at five points in each box when the tarpaulin was laid out, and were collected by pulling tied strings without removing the tarpaulin.

2.6 Sentinel birds

At the end of the litter treatments, ten 3-day old SPF chicks were housed in each experimental box (5 chicks/m^2) for 34 days, and given water and feed *ad libitum*. Chicks were daily monitored to mortality or clinical signs of illness. The chicks and litter were sampled in the first, third and fifth weeks of housing. The litter was tested for isolation of *Salmonella* spp*.;* and total enterobacteria and *Salmonella* spp*.* were enumerated using the colony-count method. At 1 and 3 weeks cloacal and tracheal swabs were taken individually for detection of IBDV (cloacal swabs) and NDV (tracheal swabs) by RT-qPCR. Cloacal swabs were also processed individually for the isolation of *Salmonella* spp.. In the fifth week the chicks were euthanized and cloacal and tracheal swabs were tested for IBDV and NDV by RT-qPCR as above. Liver and spleen, combined in individual pools, and cloacal swabs were taken for

isolation of *Salmonella* spp.. Serum samples were collected and tested for antibody titers to NDV and IBDV by enzyme-linked immunosorbent assay (ELISA).

2.7 Bacteriological analyses

For quantification of total enterobacteria and *Salmonella* spp., subsamples of 10 g of litter were homogenized in 90 mL of phosphate-buffered saline (PBS) pH 7.4, in an orbital shaker (150 rpm/10 min) and submitted to ten-fold dilutions up to 10^{-5}. Aliquots of 0.1 mL of each dilution were plated onto MacConkey agar (Acumedia, USA) for counting of total enterobacteria and onto Brilliant Green agar (BGA) (Oxoid, UK) containing novobiocin (40 mg/L), gentamicin (15 mg/L) and streptomycin (30 mg/L) for counting of *Salmonella* spp.. The plates were incubated at 37°C for 48 h. One typical *Salmonella* spp. colony was selected from each plate for confirmation through biochemical testing. The detection limit was 100 colony forming unit (CFU)/g. For isolation of *Salmonella* spp., litter samples, cloacal swabs and fragments of tissues were individually homogenized in nine volumes of 1% buffered peptone water and incubated at 37°C for 24 h. Aliquots of 0.1 and 1 mL were inoculated in Rappaport-Vassiliadis broth (Himedia, India) and tetrathionate broth (Oxoid, UK), respectively, and incubated at 42°C for 24 h. Aliquots were then streaked onto xylose lysine tergitol-4 agar (XLT4) (Difco, USA) and BGA containing novobiocin (40 mg/L), streptomycin (30 mg/L) and gentamicin (15 mg/L). The plates were incubated at 37°C for 48 h and typical colonies were submitted to confirmatory biochemical tests.

2.8 Virus isolation

NDV and IBDV were isolated in embrionated SPF chicken eggs (OIE, 2016). Cloacal and tracheal swabs harvested in 1 mL of tryptone peptone broth (TPB) were incubated with a 20% cocktail of antimicrobials containing streptomycin (50 mg/mL), gentamicin (10 mg/mL), amphotericin B (0.1 mg/mL), kanamycin (6.5 mg/mL) and penicillin G (10,000 IU) for 1 h and then inoculated into embrionated eggs of 9-11 days incubation. The eggs were incubated for 7 days at 37ºC and the embryos analyzed for the presence of specific lesions and/or haemagglutination activity. A negative result was considered after the third passage in embrionated eggs.

2.9 Total RNA extraction

A total of 40 g of each litter sample was homogenized in 160 mL of PBS (140 rpm/30 min) and refrigerated for 30 min. Then, the unsedimented suspension was clarified by centrifugation (2000 g/10 min) and aliquots were frozen at -70ºC. Total RNA was extracted with Trizol® reagent (Ambion, USA) and chloroform as indicated by manufacturer, and the total RNA contained in the aqueous phase was purified in a silica gel column (RNeasy Mini kit, Qiagen, Germany). The oropharyngeal and cloacal swabs, kept individually in 1 mL of TPB, were homogenized and kept frozen at -70ºC until processed. The total RNA was obtained by automated extraction (MagMax™ Express Magnetic Particle Processors, Applied Biosystems, USA) with a commercial kit (5X MagMax™ viral RNA Isolation Kit, Ambion, USA) and stored at -70°C.

2.10 Reverse transcription and quantitative real time polymerase chain reaction (RT-qPCR)

Total RNA extracted from samples of litter suspensions, cloacal and oropharyngeal swabs were tested individually by one step RT-qPCR in an ABI 7500 Real time PCR equipment (Applied Biosystems, USA), using a commercially available kit (AgPath-ID™ One-Step RT-PCR Kit, Ambion, USA). For NDV, a matrix gene region conserved in all avian Paramyxovirus 1 was amplified as described by Wise et al. (2004) in an assay extensively validated for official screening of NDV in poultry (OIE, 2016).

For IBDV a RT-qPCR assay was established for amplification of a fragment of the VP1 gene located in segment B of the IBDV bisegmented RNA genome. The software Primer Express® (Applied Biosystems, USA) was used to design primers (Forward 1929+ 5'GGGATCGAGCAGGCATACA3' and Reverse 1994-5'GTAGTTCCAACCACCTACCAACCT3') and probe (1951-FAM 5'TAGTCAGGTATGAGGCGTT-BHQ3') for detecting classic and variant samples of IBDV. The RNA was denatured with 25% DMSO at 92ºC for 3 min and the one-step RT-qPCR performed at 50ºC for 30 min, 95ºC for 10 min, 40 cycles of 95ºC for 15 s and 60ºC for 45 s. A calibration curve with the number of known copies of IBDV (Lukert vaccine strain) was included in the assay. To construct the calibration curve indicator primers were designed with Primer3 program: (forward 1876+ 5'GTTCTGCTGCGTATCCCAAG3' and reverse 2168-5'TTCGGCTAGGCTCTCAGATG3') and conventional RT-PCR was performed using the One-Step RT-PCR kit (Qiagen Germany) with the thermal profile of 50°C for 30 min, 95°C for 15 min and 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, final extension at 72°C for 10 min and 4°C. The amplified product was cloned in the

TOPO TA cloning kit (Invitrogen, USA). The bacterial plasmid DNA was extracted with the Miniprep kit (Qiagen, Germany), linearized with BamHI and submitted to *in vitro* run off transcription with Megascript T7 (Invitrogen, USA). Serial tenfold dilutions of the transcribed RNA were amplified by RT-qPCR and amplification cycle threshold values obtained for each dilution were used to plot a calibration curve. The number of RNA copies in the test samples was estimated by linear regression from the calibration curve. Results were interpreted as positive by RT-qPCR when the value of the Quantification Cycle (Cq) was ≤36.

2.11 Serological analyses

Serum samples were tested to specific antibody detection using ELISA commercial kits for IBDV (IDEXX IBD Ab Tests, USA) and NDV (IDEXX NDV Ab Tests, USA), according to the manufacturer's guidelines. The reading was measured with an ELISA microplate reader (BioTek, USA).

2.12 Physicochemical analyses of the broiler litter

The litter temperature was recorded every two hours during all the treatments applied by means of an automatic data recorder (iButton® DS1923, Maxim Integrated, USA) inserted in the center of the litter of each box at the beginning of the treatments. The pH was determined from litter samples suspended in ultrapure water, homogenized for 30 min and diluted at a ratio of 1:10. The dry matter content was ascertained by the difference of the drying weight of 2 g heated to 105°C for 18 h (IAL, 2008). The ammonia content ($N\text{-}NH_4^+$) was determined according to the Kjeldhal method (APHA, 2012).

2.13 Statistical analysis

For analysis of the IBDV and NDV quantification in the seeder birds, the number of viral copies was transformed to the base-10 log scale. The mean averages were calculated for each experimental unit (box) and analysis of variance was performed for the completely randomized design model. Whenever the F test detected a significant effect ($p \leq 0.05$) of treatments, drilldown analysis was conducted by means of the protected t test for multiple comparisons of means. Determination of total enterobacteria level, dry matter, pH, ammonia content and temperature of the litter was analyzed using the theory of mixed models for repeated measures, considering the treatment effects, days of treatment and interaction of these factors, and 15 types of variance and covariance matrix structures, using PROC MIXED (SAS, 2012), according to Xavier (2000). The structure used in the analysis was chosen based on the lowest value of the Akaike Information Criterion (AIC). The maximum likelihood estimation method was used. Breakdown of the effect of treatments was performed by means of the protected t test whenever the F test detected significant effect ($p \leq 0.05$) of the treatment. The total enterobacteria quantification data were transformed into base-10 log for analysis. For qualitative evaluations of *Salmonella* spp., IBDV and NDV Fisher's exact test was applied to compare the treatments on each day of the evaluation.

3. Results

3.1 Method of viral contamination of the litter

Analysis of the cloacal swabs demonstrated that IBDV shed by seeder birds increased each day of the evaluation, whereas for NDV the frequency of positive oropharyngeal swabs reduced from the fifth day onwards (Figure 1). In terms of

allowing the highest levels of viral replication, the optimum housing time of the seeder birds, inoculated simultaneously with IBDV and NDV, was between days 5 and 7 pi. The method of contamination by seeder birds allowed detection of both viruses in the litter by RT-qPCR and viral isolation, where IBDV was detected on days 3, 5 and 7 pi (3.6; 3.4 and 3.2 $\log_{10}$, respectively) and NDV only on days 3 and 5 pi (2.3 and 1.4 $\log_{10}$, respectively). Furthermore, after exposure to the contaminated litter by the seeder bird method, 60% (6/10) of the sentinel chicks tested positive for IBDV and all tested negative for NDV. On the other hand, by the direct spray method only IBDV was detected in the broiler litter in the evaluated period, and all sentinel chicks tested negative for both viruses.

3.2 Confirmation of experimental contamination

Viral replication in the seeder birds used to contaminate the broiler litter was confirmed by RT-qPCR in all the experimental units from T1 to T4, with average RNA copy numbers of 1.21±0.13 $\log_{10}$ for IBDV and 2.33±0.14 $\log_{10}$ for NDV. Initial contamination of the litter with *S.* Heidelberg from T1 to T4 was confirmed by microbiological isolation, whose average levels were lower than the detection limit in the quantitative analysis (100 CFU/g).

3.3 Effect of the treatments on the broiler litter

The results of the total enterobacteria level, pH, dry matter, ammonia content, and temperature measured in litter during the treatment period are presented in Table 1. A significant effect (p<0.05) was identified in all the factors for all variables on at least one evaluation day. *S.* Heidelberg was detected in the litter in T1, T2, T3

and T4 on every assessment day. On the other hand, T5 (uncontaminated fresh litter) remained negative throughout the experimental period (data not shown).

3.4 Evaluation of sentinel birds housed on the litter

No clinical sign of illness or mortality was observed in any of the sentinel chicks during the experiment in all the evaluated treatments. Figure 2 presents the percentages of sentinel birds tested positive for *S.* Heidelberg and IBDV during housing on the contaminated litter submitted to T1 to T4. The sentinel birds tested for NDV did not present seroconversion, and tested negative in the RT-qPCR analyses in all the treatments. In T2, the IBDV-positive sentinel birds presented average RNA copies of 3.71±1.20 and 2.02±0.86 $\log_{10}$ in the third and fifth weeks, respectively. In T4, these means were 3.34±1.40 and 2.39±0.97 $\log_{10}$, respectively. The sentinel birds housed on the fresh, uncontaminated litter (T5) remained negative throughout the experimental period (data not shown), confirming that no cross contamination occurred between the evaluated treatments.

4. Discussion

4.1 Methods of litter viral contamination and assessment of sentinel birds

In this study, viral shedding promoted by seeder birds was more effective than the direct spraying in establishing litter viral contamination. The efficacy of the applied treatments was evaluated using a bioassay method (Islam et al., 2013; Crespo et al., 2016), in which the infectivity of the remaining microorganisms in the treated broiler litter was investigated through the use of sentinel birds, allowing the

treatment efficiency to be directly inferred in the host. This method especially favored viral detection in the litter in view of the limitations imposed by other techniques in differentiating infective and non-viable viruses and the limitations to isolate virus from highly contaminated environments (Guan et al., 2010; Islam et al., 2013).

4.2 Effect of the litter treatments on microorganisms

The result of the sentinel chicks in view of the RT-qPCR and ELISA tests to IBDV revealed that T1 and T3 inactivated this virus in the broiler litter. The sentinel chicks of all the treatments were negative for IBDV by RT-qPCR in the first week. Likewise, Crespo et al. (2016) detected IBDV in sentinel birds only after ten days of exposure to the contaminated litter and on the 12^{th} day 30% of the birds tested positive. In this study, from the third week onwards the virus was detected in 50% and 40% of the sentinel chicks of T2 and T4, respectively, leading 100% of the birds to seroconversion by the fifth week. IBDV is notoriously highly contagious (Müller et al., 2012), as we observed in T2 and T4. Therefore, absence of the virus in the sentinel chicks of T1 and T3 demonstrated that these treatments inactivated IBDV in the litter. This result is important, firstly because it indicates that shallow fermentation is able to inactivate IBDV, which is highly resistant to environmental conditions (Müller et al., 2003; Islam et al., 2013; Crespo et al., 2016). Hence, this treatment could be an option to avoid the persistence of IBDV in broiler litter after episodes of the disease. Secondly, because it suggests that shallow fermentation also has an effect on other less resistant viruses in the litter (Guan et al., 2010). There was no difference between T1 and T3 in terms of viral inactivation; however, T1 was significantly superior to T3 in reducing total enterobacteria at the end of the litter treatment. This result indicates that the association of quicklime with shallow

fermentation did not enhance the treatment effect. In a previous study, T1 was also superior in reducing total enterobacteria, whereas T2 did not differ from the untreated group (Vaz et al., 2017). Studies with the addition of quicklime in the recycled litter have presented contradictory results in relation to antimicrobial activity (Bennett et al., 2003; Bennett et al., 2005; Ruiz et al., 2008; Vaz et al., 2017; Lopes et al., 2015). This variability may be due to different time of treatment and/or concentration of quicklime used (Bennett et al., 2005). Bennett et al. (2003) observed inhibitory action of the hydrated quicklime on *Salmonella* in concentrations of 5, 10 and 20%; however, this result was not obtained when lower concentrations (0, 2, 1 and 5%) were applied in the contaminated broiler litter (Bennett et al., 2005). Variation in the height and/or density of the litter also hinder comparisons between experiments. Fifth flock litter can be twice as dense as first flock litter (Loch et al., 2011).

In this study, *S.* Heidelberg was detected in the broiler litter in all the treatments evaluated. In a previous study, T1, T2 and windrowing were able to inactivate *S.* Enteritidis in recycled litter (Vaz et al., 2017). However, the particular characteristics of each serovar or strain may influence the survival of *Salmonella* spp. in distinct niches (Chen et al., 2014; Andino et al., 2014), and therefore it is not surprising that the different *Salmonella* serovars respond differently to the same litter treatment. Detection of *S.* Heidelberg in sentinel chicks after exposure to contaminated litter revealed that broiler litter is a potential source of contamination for the subsequent flock. For this reason, other strategies are required to inactivate residual *S.* Heidelberg in recycled broiler litter.

NDV failed to survive in the litter, regardless of the treatment applied. Elevating the inoculation dosage for the seeder birds resulted in higher titers of the virus in the chicks, but it did not remain viable in the litter. Single-stranded RNA

viruses are also known as being more sensitive to environmental conditions (Decrey et al., 2016). Reis et al. (2012) evaluated the tenacity of the avian influenza virus, another RNA virus, which only remained infective in the litter for up to three days. Other authors have also described that the vaccine strain of NDV shed by chickens is not readily transmitted through broiler litter (Islam et al., 2013) or survived for less than one week, being more rapidly inactivated in the litter than in other matrixes (Guan et al., 2009).

4.3 Physicochemical factors and antimicrobial activity

Physicochemical factors such as pH, temperature, moisture and ammonia contents interfere with the viability of microorganisms in the environment (Turnbull and Snoeyenbos, 1973; Islam et al., 2013; Chen et al., 2015; Magri et al., 2015). In this study, the ammonia content in the litter in T1 and T3 increased as from the 6th day in relation to the other treatments (Table 1) and this increase was associated to the observed antimicrobial effect. The effect of ammonia on *Salmonella* spp*.* is known (Turnbull and Snoeyenbos, 1973; Himathongkham and Riemann, 1999; Park and Diez-Gonzalez, 2003; Chen et al., 2015) as its virucidal activity (Ward, 1978; Scodeller et al., 1984; Magri et al., 2015; Decrey et al., 2016) but the mechanism involved is not completely clear (Chen et al., 2015). Scodeller et al. (1984) identified that the ionic strength of NH_4 activated a viral endonuclease that degraded the foot-and-mouth disease virus genome. It is also believed that NH_3 causes a rapid alkalization of the cytoplasm because is passes through the cell membrane by simple diffusion and reduces the proton concentration (Park and Diez-Gonzalez, 2003) promoting alkaline hydrolysis and degradation of the viral RNA (Ward, 1978). Furthermore, the tarpaulin cover acts like an impermeable barrier that allows

ammonia to accumulate in the litter and favors the reduction of microorganisms (Macklin et al., 2006).

On the other hand, the range of temperatures measured during the treatments (12.6 – 25.1°C) did not reach levels considered critical for the microorganisms evaluated (Macklin et al., 2006; Guan et al., 2009). Similarly, the pH and moisture content (interpreted as the inverse of dry matter content) exerted no detectable inhibitory activity on the microorganisms evaluated. In T1 and T3 the addition of water to the litter prior to the tarpaulin covering resulted in lower levels of dry matter and, despite this, these treatments were more effective on microbial reduction. Additionally, although in T2 and T3 the pH and dry matter content increased on day 12 with the addition of quicklime, T2 failed to inhibit the evaluated microorganisms. Quicklime has a drying action and its antimicrobial activity results from the reduction of available water associated to the increased pH of the litter (Ruiz et al., 2008). In a previous study, quicklime applied for the same period of time to the broiler litter, however in a lower concentration, resulted in pH 9.6, eliminating *S.* Enteritidis but not reducing the level of total enterobacteria (Vaz et al., 2017). In the present study, the concentration of quicklime was high (600g/m^2), resulting in a higher pH level (10) which, however, was not reflected in microbial reduction. In the USA, when quicklime is the option used for microbial reduction in sewage sludge, the rule requires that sufficient quicklime is added to raise the pH to 12 for 2 h to kill pathogens (EPA, 1999). However, the concentration of quicklime used in this study, and even the natural buffering activity of the litter (Cassity-Duffey et al., 2015), did not allow reached this pH level.

5. Conclusion

The shallow fermentation treatment eliminated IBDV and reduced total enterobacteria in the recycled litter, but had no effect on *S.* Heidelberg. The NDV did not survive in the broiler litter, regardless of the treatment applied. The antimicrobial activity was related to higher ammonia content in the broiler litter in relation to the other treatments evaluated. The use of this treatment in litter recycled between broiler flocks should be complemented by another control strategy to inactivate *S.* Heidelberg.

Acknowledgement

This work was supported by the Brazilian Agricultural Research Corporation - Embrapa, grant number 0313100900. The authors are grateful to Tânia A. P. Klein for her technical assistance.

References

Andino, A., Pendleton, S., Zhang, N., Chen, W., Critzer, F., Hanning, I., 2014. Survival of *Salmonella enterica* in poultry feed is strain dependent. Poult. Sci. 93, 441-447. DOI: 10.3382/ps.2013-03401.

APHA (American Public Health Association), 2012. Standard Methods for the Examination of Water and Wastewaters. Nitrogen (Ammonia) (APHA 4500-NH3).

Bennett, D.S., Higgins, S.E., Moore, R., Byrd, J.A., Beltran, R., Corsiglia, C., Caldwell, D., Hargis, B.M., 2005. Effect of addition of hydrated lime to litter on

recovery of selected bacteria and poultry performance. J. Appl. Poult. Res. 14, 721-727. DOI: 10.1093/japr/14.4.721.

Cassity-Duffey, K., Cabrera, M., Mowrer, J., Kissel, D., 2015. Titration and Spectroscopic Measurements of Poultry Litter pH Buffering Capacity. J. Environ. Qual. 44, 1283-1292. DOI:10.2134/jeq2014.11.0463.

Chen, W., Golden, D.A., Critzer, F.J., 2014. Salmonella survival and differential expression of fatty acid biosynthesis-associated genes in a low-water-activity food. Lett. Appl. Microbiol. 59, 133-138. DOI: 10.1111/lam.12253.

Chen, Z., Wang, H., Ionita, C., Luo, F., Jiang, X., 2015. Effects of chicken litter storage time and ammonia content on thermal resistance of desiccation-adapted *Salmonella* spp. Appl. Environ. Microbiol. 81, 6883-6889. DOI: 10.1128/AEM.01876-15.

Crespo, R., Badcoe, L.M., Williams, C., Bary, A.I., 2016. Inactivation of infectious Bursal Disease Virus through composting of litter from poultry houses. Avian Dis. 60, 506–510. DOI: 10.1637/11341-120615-ResNote.

Decrey, L., Kazama, S., Kohn, T., 2016. Ammonia as an in situ sanitizer: influence of virus genome type on inactivation. Appl. Environ. Microbiol. 82, 4909-4920. DOI: 10.1128/AEM.01106-16.

EPA (US Environmental Protection Agency), 1999. Biosolids generation, use, and disposal in the United States. US EPA, Office of Wastewater Management, Washington, DC.

Fleischmann, W.R. Jr. 1996. Viral Genetics. In: Baron S. (Ed). Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; Chapter 43. Available from: https://www.ncbi.nlm.nih.gov/books/NBK8439/

Guan, J., Chan, M., Grenier, C., Wilkie, D.C., Brooks, B.W., Spencer J.L., 2009. Survival of Avian Influenza and Newcastle Disease Viruses in Compost and at Ambient Temperatures Based on Virus Isolation and Real-Time Reverse Transcriptase PCR. Avian Dis. 53, 26-33. DOI: 10.1637/8381-062008-Reg.1.

Guan, J., Chan, M., Brooks, B.W., Spencer J.L., 2010. Infectious bursal disease virus as a surrogate for studies on survival of various poultry viruses in compost. Avian Dis. 54, 919-922. DOI: 10.1637/9115-102209-ResNote.1.

Himathongkham, S., Riemann, H., 1999. Destruction of *Salmonella typhimurium*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* in chicken manure by drying and/or gassing with ammonia. FEMS Microbiol. Lett. 171, 179-182.

IAL (Instituto Adolfo Lutz), 2008. Métodos físico-químicos para análise de alimentos. Zenebon O., Pascuet N.S., Tiglea P. (Ed.) São Paulo: IAL. Método IAL 012/IV, pp. 98. Avaliable from: http://www.crq4.org.br/sms/files/file/analisedealimentosial_2008.pdf

Islam, A.F.M.L., Walkden-Brown, S.W., Groves, P.J., Wells, B., 2013. Development of a chick bioassay for determination of infectivity of viral pathogens in poultry litter. Aust. Vet. J. 91, 65-71. DOI: 10.1111/avj.12010.

Loch, F.C., Oliveira, M.C., Silva, D., Gonçalves, B.N., Faria, B.F., Menezes, J.F.S., 2011. Quality of poultry litter submitted to different treatments in five consecutive flocks. Rev. Bras. Zootec. 40, 1025-1030. DOI: 10.1590/S1516-35982011000500013.

Lopes, M., Leite, F.L., Valente, B.S., Heres, T., Dai Prá, M.A., Xavier, E.G., Roll, V.F.B., 2015. An assessment of the effectiveness of four in-house treatments to reduce the bacterial levels in poultry litter. Poult. Sci. 00, 1-5. DOI: 10.3382/ps/pev195.

Macklin, K.S., Hess, J.B., Bilgili, S.F., Norton, R.A., 2006. Effects of in-house composting of litter on bacterial levels. J. Appl. Poult. Res. 15, 531-537.

Magri, M.E., Fidjeland, J., Jönsson, H., Albihn, A., Vinnerás, B., 2015. Inactivation of adenovirus, reovirus and bacteriophages in fecal sludge by pH and ammonia. Sci. Total Environ. 520, 213-221. DOI: 10.1016/j.scitotenv.2015.03.035.

Müller, H., Islam, M.R., Raue, R., 2003. Research on infectious bursal disease - the past, the present and the future. Vet. Microbiol. 97, 153-165.

Müller, H., Mundt, E., Eterradossi, N., Islam, M.R., 2012. Current status of vaccines against infectious bursal disease. Avian Pathol., 41, 133-139. DOI: 10.1080/03079457.2012.661403.

OIE (World Organization for Animal Hearth), 2016. Manual of diagnostic tests and vaccines for terrestrial animals 2016. Chapter 2.3.12 Infectious Bursal Disease and Chapter 2.3.14 Newcastle Disease. Avaliable from: http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/

Park, G.W., Diez-Gonzalez, F., 2003. Utilization of carbonate and ammonia-based treatments to eliminate Escherichia coli O157:H7 and *Salmonella* Typhimurium DT104 from cattle manure. J. Appl. Microbiol. 94, 675-685.

Pulido-Landínez, M., Sanchez-Ingunza, R., Guard, J., do Nascimento, V.P., 2013. Assignment of serotype to *Salmonella enterica* isolates obtained from poultry and their environment in southern Brazil. Lett. Appl. Microbiol. 57, 288-294. DOI: 10.1111/lam.12110.

Reis, A., Stallknecht, D., Ritz, C., García, M., 2012. Tenacity of low-pathogenic avian influenza viruses in different types of poultry litter. Poult. Sci. 91, 1745-1750.

Ruiz, V., Ruiz, D., Gernat, A.G., Grimes, J.L., Murillo, J.G., Wineland, M.J., Anderson, K.E., Maguire, R.O., 2008. The effect of quicklime (CaO) on litter condition and broiler performance. Poult. Sci. 87, 823-827. DOI: 10.3382/ps.2011-01625.

Scodeller, E.A., Lebendiker, M.A., Dubra, M.S., Crespo, O.A., Basarab, O., La Torre, J.L., Vasquez, C., 1984. Inactivation of foot-and-mouth disease virus vaccine strains by activation of virus-associated endonuclease. J. Gen. Virol. 65, 1567-1573. DOI: 10.1099/0022-1317-65-9-1567.

Shivaprasad, H.L., Methner, U., Barrow, P.A., 2013. *Salmonella* infections in the domestic fowl. In: Barrow, P.A., Methner, U. *Salmonella* in domestic animals, 2nd edn, CABI, Boston, pp. 162-192.

Torok, V.A., Hughes, R.J., Ophel-Keller, K., Ali, M., MacAlpine, R., 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poult. Sci. 88, 2474-2481. DOI: 10.3382/ps.2008-00381.

Turnbull, P.C, Snoeyenbos, G.H., 1973. The roles of ammonia, water activity, and pH in the salmonellacidal efect of long-used poultry litter. Avian Dis. 17, 72-86.

Vaz, C.S.L., Voss, D., Avila, V.S., Coldebella, A., Silva, V.S., 2017. Interventions to reduce the bacterial load in recycled broiler litter. Poult. Sci. (in press).

Voss-Rech, D., Vaz, C.S.L., Alves, L., Coldebella, A., Leão, J.A., Rodrigues, D.P., Back, A., 2015. A temporal study of *Salmonella enterica* serotypes from broiler farms in Brazil. Poult. Sci. 94, 433-441. DOI: 10.3382/ps/peu081.

Xavier, L.H., 2000. Univariate and multivariate models for analysis of repeated measures and verification of the accuracy of the univariate model through simulation. Masters thesis. Escola Superior de Agricultura Luiz de Queiroz (ESALQ), Universidade de São Paulo (USP), Piracicaba.

Ward, R.L., 1978. Mechanism of poliovirus inactivation by ammonia. J. Virol. 26, 299-305.

Wise, M.G., Suarez, D.L., Pedersen, J.C., Senne, D.A., King, D.J., Kapczynski, D., Spackman, E., 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. J. Clin. Microbiol. 42, 329-338.

Table 1. Means and standard errors of the variables measured in the broiler litter according to treatments and time of treatment.

Time (days)	Treatment				Pr>F
	1	2	3	4	
	Total enterobacteria (log_{10} CFU/g)				
0	5.662±0.210	5.091±0.427	5.864±0.150	5.005±0.146	0.2747
6	4.096±0.429	4.875±0.217	5.106±0.321	4.919±0.260	0.2289
12	4.469±0.309	4.062±0.597	4.898±0.304	4.742±0.139	0.3981
14	3.435±0.254 [a]	4.921±0.430 [b]	4.375±0.256 [ab]	5.282±0.735 [b]	0.0060
	Dry matter (%)				
0	73.10±0.66 [b]	79.52±0.38 [a]	73.24±0.70 [b]	78.91±0.51 [a]	<.0001
6	72.16±0.73 [b]	82.94±0.44 [a]	71.57±0.97 [b]	82.68±0.25 [a]	<.0001
12	73.96±0.65 [b]	82.45±0.33 [a]	73.61±0.46 [b]	81.13±0.38 [a]	<.0001
14	74.33±0.26 [c]	81.86±0.35 [a]	74.96±0.37 [c]	80.87±0.27 [b]	<.0001
	$N\text{-}NH_4^+$ (mg/Kg)				
0	1520±60 [bc]	1483±60 [c]	1646±47 [ab]	1705±52 [a]	0.0221
6	2767±170 [a]	1256±68 [b]	2751±176 [a]	1319±68 [b]	<.0001
12	2828±163 [a]	1218±25 [b]	2541±122 [a]	1455±106 [b]	<.0001
14	2178±94 [a]	1053±25 [c]	1742±127 [b]	1175±26 [c]	<.0001
	Temperature (ºC)				
0	21.40±0.23 [a]	20.80±0.11 [b]	21.37±0.13 [a]	19.93±0.21 [c]	0.0002
6	19.38±0.37 [a]	17.07±0.20 [b]	19.47±0.33 [a]	15.46±0.62 [c]	<.0001
12	18.17±0.26 [a]	17.03±0.22 [b]	18.67±0.28 [a]	15.55±0.42 [c]	<.0001
14	19.09±0.22 [a]	18.98±0.26 [a]	19.48±0.18 [a]	17.48±0.31 [b]	0.0005
	pH				
0	8.690±0.026	8.558±0.046	8.545±0.027	8.563±0.060	0.1901
6	8.693±0.095	8.593±0.027	8.695±0.057	8.585±0.043	0.2776
12	8.858±0.013 [c]	10.01±0.06 [a]	9.423±0.067 [b]	8.530±0.052 [d]	<.0001
14	8.825±0.009 [b]	9.275±0.063 [a]	9.303±0.036 [a]	8.488±0.074 [c]	<.0001

Averages followed by distinct letters on the rows differ significantly by the t test ($p \leq 0.05$).

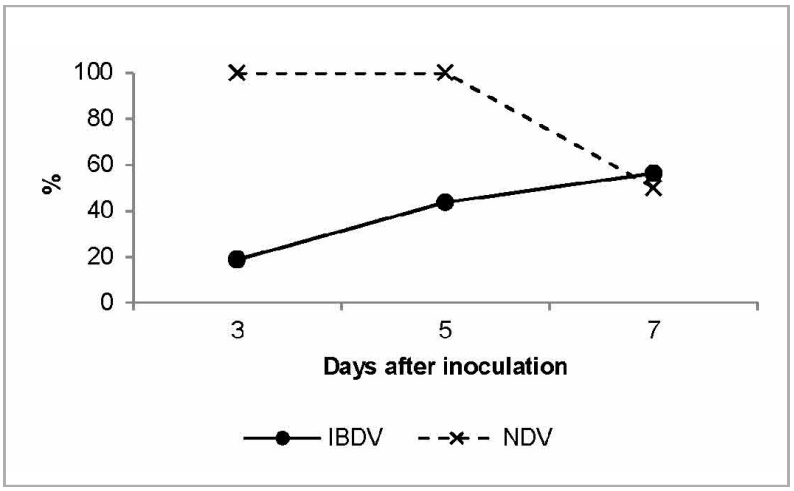


Figure 1. Frequency of IBDV- and NDV-positive seeder birds, by the RT-qPCR test of cloacal and tracheal swabs, respectively.

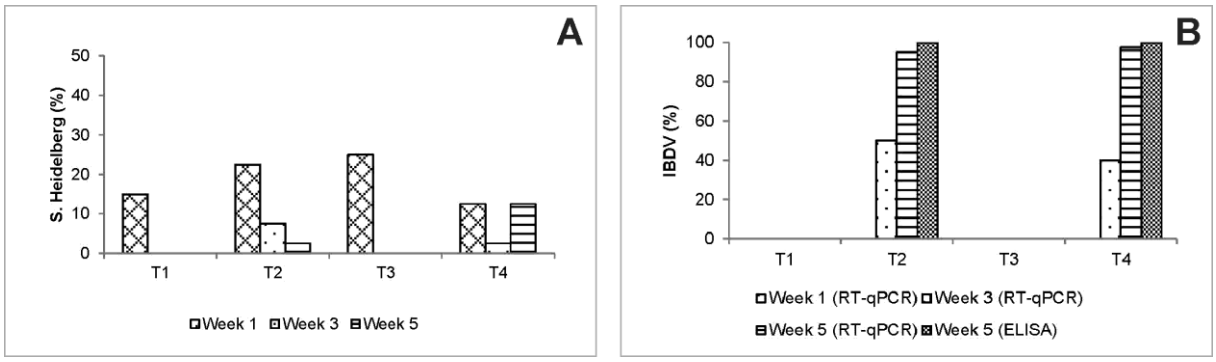
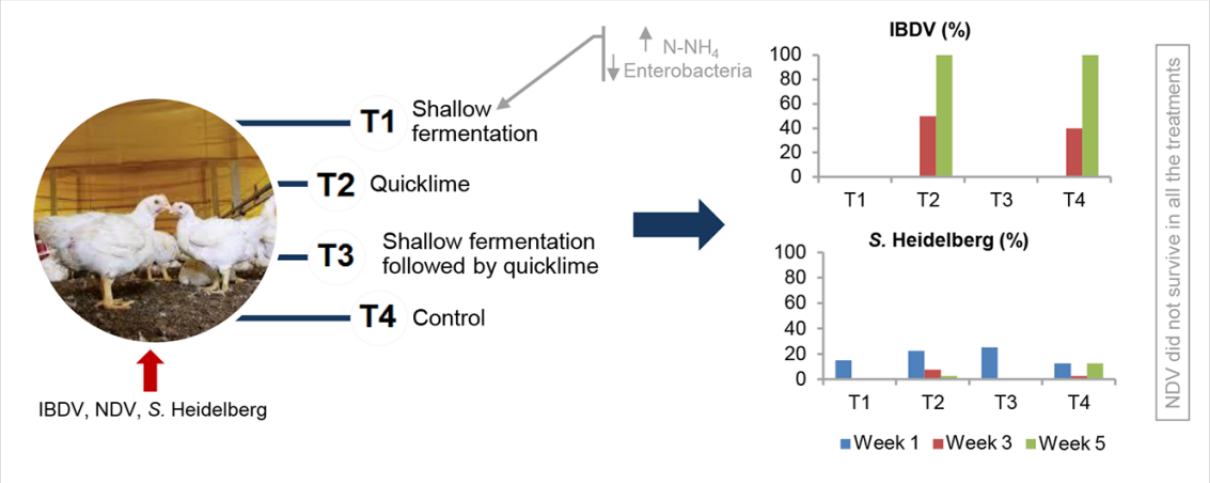


Figure 2. Frequency of *S.* Heidelberg-positive sentinel chicks (A) in bacteriological isolation and IBDV-positive sentinel chicks (B) by RT-qPCR and ELISA tests.

Highlights

- Residual Infectious Bursal Disease Virus (IBDV) and *Salmonella* Heidelberg remained infective in the recycled broiler litter;
- The shallow fermentation treatment inactivated IBDV and reduced total enterobacteria levels in the broiler litter;
- The antimicrobial activity of shallow fermentation was related to the ammonia content of the litter;
- *S.* Heidelberg resisted to all litter treatments evaluated.

Abstract Graphical



5 CONCLUSÃO

O tratamento de fermentação plana eliminou o VDIB e reduziu enterobactérias totais na cama aviária reutilizada; todavia, não foi verificado efeito sobre *S.* Heidelberg. A ação antimicrobiana deste tratamento pode ser relacionada aos maiores teores de amônia presentes na cama aviária em relação aos demais tratamentos avaliados. O VDNC não sobreviveu na cama aviária, independente do tratamento aplicado. O uso da fermentação plana para o tratamento da cama aviária reutilizada entre lotes deve ser complementado por outra estratégia de controle na presença de *S.* Heidelberg. Adicionalmente, observou-se que a contaminação experimental da cama aviária pelos vírus aviários foi mais eficiente com o uso de *seeder birds*, quando comparada à aspersão direta na cama. A avaliação de aves sentinelas apresentou-se uma estratégia adequada para monitorar a eficiência dos tratamentos de cama sobre os micro-organismos estudados.

REFERENCIAS

ÁVILA, V. S. de et al. **Boas práticas de produção de frangos de corte**. Embrapa Suínos e Aves, Concórdia, SC, 2007. 28 p. (Circular Técnica, 51).

ANDINO, A.; HANNING, I. *Salmonella enterica*: Survival, Colonization, and Virulence Differences among Serovars. **Scientific World J.**, 2015, Article ID 520179, 16 p. 2015.

BERNARDINO, A.; LEFFER, E. Doença infecciosa da bolsa de Fabrício. In: BERCHIERI JÚNIOR, A. et al. (Ed.). **Doenças das aves.** Campinas, SP: Fundação APINCO de Ciência e Tecnologia Avícolas, 2009. p. 651-673.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Instrução Normativa n.º 8, de 25 de março de 2004. Proíbe em todo o território nacional a produção, a comercialização e a utilização de produtos destinados à alimentação de ruminantes que contenham em sua composição proteínas e gorduras de origem animal. **D.O.U.,** Brasília, DF, 26 de mar. 2004. Seção 1, p. 5.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Instrução Normativa n° 17, de 7 de abril de 2006. Aprova, no âmbito do Programa Nacional de Sanidade Avícola, o Plano Nacional de Prevenção da Influenza Aviária e de Controle e Prevenção da Doença de Newcastle. **D.O.U.,** Brasília, DF, 10 de mai. 2006, Seção 1, p. 6.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Instrução Normativa n° 10, de 11 de abril de 2013. Define o programa de gestão de risco diferenciado para estabelecimentos avícolas. **D.O.U.,** Brasília, DF, 12 de abr. 2013, Seção 1, p. 2.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Instrução Normativa n° 21, de 21 de outubro de 2014. Estabelece as normas técnicas de Certificação Sanitária da Compartimentação da Cadeia Produtiva Avícola das granjas de reprodução, de corte e incubatórios para a infecção pelos vírus de influenza aviária e doença de Newcastle. **D.O.U.,** Brasília, DF, 22 de out. 2014, Seção 1, p. 4.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa n° 20, de 21 de outubro de 2016. Estabelece o controle e o monitoramento de *Salmonella spp*. nos estabelecimentos avícolas comerciais de frangos e perus de corte e nos estabelecimentos de abate de frangos, galinhas, perus de corte e reprodução, registrados no Serviço de Inspeção Federal (SIF). **D.O.U.**, Brasília, DF, 25 de out. 2016. Seção 1, p. 13.

BRACKE, M.B.M.; HOPSTER, H. Assessing the importance of natural behavior for animal welfare. **J. Agric. Environ. Ethics**, v. 19, n. 1, p. 77-89, 2006.

CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC). Outbreak of Salmonella Heidelberg infections linked to a single poultry producer – 13 states, 2012-2013. **Morb. Mort. Weekly Rep.** 62, 2013. p. 553- 556.

CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC). Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet 2015 (Preliminary Data). Atlanta, Georgia: U.S. Department of Health and Human Services, CDC, 2015.

CORRIER, D. E.; HARGIS, B. M.; HINTON Jr., A. Effect of used litter from floor pens of adult broilers on *Salmonella* colonization of broiler chicks. **Avian Dis.**, [Jacksonville], v. 36, n. 4, p. 897-902, 1992.

CRESPO, R. et al. Inactivation of infectious Bursal Disease Virus through composting of litter from poultry houses. **Avian Dis.**, [Jacksonville], v. 60, n. 2, p. 506–510, 2016.

CRESSMAN, M. D. et al. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. **Appl. Environ. Microbiol.**, [Washington], v. 76, n.19, p. 6572-6582, 2010.

DAVIES, R. H.; WRAY, C. Persistence of *Salmonella* Enteritidis in poultry units and poultry food. **Br. Poult. Sci.**, [London], v. 37, n. 3, p. 589-596, 1996.

ENTERADOSSI, N.; SAIF, Y. Infectious bursal disease. In: SAIF Y. (ed.). **Diseases of poultry**. 12 ed. Ames: Iowa State University Press, 2008. p. 185-208.

EUROPEAN FOOD SAFETY AUTHORITY (EFSA); EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL (ECDC). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. **EFSA J.**, Parma, v. 13, n. 2, 2015. 4329 p. Disponível em: <http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2015.4329/epdf>. Acesso em: 08 nov. 2016.

FIORENTIN, L. Processos de tratamento para reutilização de cama de aviário: aspectos bacteriológicos. In: CONFERÊNCIA APINCO DE CIÊNCIA E TECNOLOGIA AVÍCOLAS, 2006, Santos. **Anais**...Campinas: FACTA, 2006.

GLOBALG.A.P. **Integrated Farm Assurance Livestock Base.** Benchmarking Cross-Reference Checklist: Control Points and Compliance Criteria - Poultry. 5.0-2 ed. Cologne, Germany, 2016.

GUAN, J. et al. Infectious bursal disease virus as a surrogate for studies on survival of various poultry viruses in compost. **Avian Dis.**, [Jacksonville], v. 54, n. 2, p. 919-922, 2010.

HIGGINS, S. E. et al. Evaluation of a Lactobacillus-based probiotic culture for the reduction of *Salmonella* Enteritidis in neonatal broiler chicks. **Poult. Sci.**, [Oxford], v. 87, n. 1, p. 27-31, 2008.

ISLAM, A. F. M. L. et al. Development of a chick bioassay for determination of infectivity of viral pathogens in poultry litter. **Aust. Vet. J.**, v. 91, n. 1-2, p. 65-71, 2013.

JONES, F. T.; HAGLER, W. M. Observations on new and reused litter for growing broilers. **Poult. Sci.**, [Oxford], v. 62, p. 175-179, 1983.

LOPES, M. et al. An assessment of the effectiveness of four in-house treatments to reduce the bacterial levels in poultry litter. **Poult. Sci**., [Oxford], v. 00, p. 1-5, 2015.

LU, J. et al. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. **Appl. Environ. Microbiol.**, [Washington], v. 69, n. 2, p. 901-908, 2003.

MACKLIN, K. S. et al. Effects of in-house composting of litter on bacterial levels. **J. Appl. Poult. Res.**, [Oxford], v. 15, n. 4, p. 531-537, 2006.

MARCOLIN, S. Processos de tratamento para reutilização de cama de aviário: Aspectos econômicos. In: CONFERÊNCIA APINCO DE CIÊNCIA E TECNOLOGIA AVÍCOLAS, 2006, Santos. **Anais**...Campinas: FACTA, 2006.

MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO (MAPA). Aves. Brasília, 2014. Disponível em: <http://www.agricultura.gov.br/animal/especies/aves>. Acesso em: 28 jun. 2014.

MÜLLER, H.; ISLAM, M. R.; RAUE, R. Research on infectious bursal disease - the past, the present and the future. **Vet. Microbiol.**, [Amsterdam], v. 97, n.1-2, p. 153-165, 2003.

MÜLLER, H. et al. Current status of vaccines against infectious bursal disease, **Avian Pathol.**, [London], v. 41, n.2, p. 133-139, 2012.

OVIEDO-RONDON, E. O. Molecular methods to evaluate effects of feed additives and nutrients in poultry gut microflora. **Rev. Bras. Zootec.**, v. 38, n. spe, p. 209-225, Viçosa, 2009.

RAPID ALERT SYSTEM FOR FOOD AND FEED (RASFF). RASFF Portal. European Commission. Brussels, 2015. Disponível em: <http://ec.europa.eu/food/safety/rasff/portal/index_en.htm>. Acesso em: 27 mar. 2015.

RANI, S.; KUMAR, S. Evaluation of infectious bursal disease virus stability at different conditions of temperature and pH. **Biologicals**. v. 46, n. 3. p. 515-518. 2015.

RITZ C. W.; FAIRCHILD B. D.; LACY M. P. **Litter quality and broiler performance**. Cooperative Extension Service, University of Georgia (UGA), College of Agricultural and Environmental Sciences. Georgia, 2014. (UGA Extension

Bulletin 1267). Disponível em: <http://extension.uga.edu/publications/files/pdf/B%201267_4.PDF>. Acesso em: 08 nov. 2016.

ROLL, V. F. B.; DAI PRÁ, M. A.; ROLL, A. P. Research on *Salmonella* in broiler litter reused for up to 14 consecutive flocks. **Poult. Sci.**, [Oxford], v. 90, n. 10, p. 2257-2262, 2011.

ROSA, P. S. et al. Cama para frangos de corte. In: MACARI, M. et al. (Ed.). **Produção de frangos de corte**, 2 ed. FACTA: Campinas, cap. 9, p. 153-180. 2014.

ROTHROCK, M. J. Jr. et al. The characterization of *Salmonella* enterica serotypes isolated from the scalder tank water of a commercial poultry processing plant: recovery of a multidrug-resistant Heidelberg serovar. **Poult. Sci.**, [Oxford], v. 94, n. 3, p. 467-472, 2015.

SILVA, V. S. et al. **Efeito de tratamentos sobre a carga bacteriana de cama de aviário reutilizada em frangos de corte**. Embrapa Suínos e Aves, Concórdia, SC, 2007. 10 p. (Comunicado Técnico 467).

SILVA, V. S. et al. Broiler litter treatment effects on enterobacteria and *Salmonella* Enteritidis reduction levels for bedding reusing: a Brazilian experience. In: WORLD VETERINARY POULTRY ASSOCIATION CONGRESS, 2009. Marrakesh. **Procedings**… Marrakesh: WVPA, p.143, 2009.

SHIVAPRASAD, H. L.; METHNER, U.; BARROW, P. A. *Salmonella* infections in the domestic fowl. In: BARROW, P. A.; METHNER, U. (Ed.) ***Salmonella* in domestic animals**, 2º ed. Boston: CABI, 2013. cap. 9, p. 162-192.

UNITED STATES DEPARTMENT OF AGRICULTURE (USDA), Food Safety and Inspection Service (FSIS). Serotypes of *Salmonella* isolates from meat and poultry products: January 1998 through December 2014. Washington, 2016. Disponível em: <https://www.fsis.usda.gov/wps/portal/fsis/topics/data-collection-and-reports/microbiology/annual-serotyping-reports>. Acesso em: 08 nov. 2016.

VOSS-RECH, D. et al. A temporal study of *Salmonella enterica* serotypes from broiler farms in Brazil. **Poult. Sci.**, [Oxford], v. 94, n. 3, p. 433-441, 2015.

WORLD ORGANIZATION FOR ANIMAL HEARTH (OIE). Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.3.14. Newcastle Disease. Paris, 2016. Disponível em: <http://www.oie.int/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals/>. Acesso em: 08 nov. 2016.

WILLIAMS, J. E.; BENSON, S. T. Survival of *Salmonella* typhimurium in poultry feed and litter at three temperatures. **Avian Dis.**, p. 742-747, 1978.

ZIPRIN, R. L. et al. Colonization control of lactose-fermenting *Salmonella* Typhimurium in young broiler chickens by use of dietary lactose. **Am. J. Vet. Res**., [Schaumburg], v. 52, n. 6, p. 833–837, 1991.

APÊNDICE A – ARTIGO ADICIONAL DESENVOLVIDO NO MESTRADO E ACEITO PARA PUBLICAÇÃO NA REVISTA FOODBORNE PATHOGENS AND DISEASES (FPD) (ISSN 1556-7125)

FOODBORNE PATHOGENS AND DISEASE
Volume 14, Number 2, 2017

DOI: 10.1089/fpd.2016.2228

Antimicrobial Resistance in Nontyphoidal *Salmonella* Isolated from Human and Poultry-Related Samples in Brazil: 20-Year Meta-Analysis

Daiane Voss-Rech,[1,2] Luciana Potter,[3] Clarissa Silveira Luiz Vaz,[2] Daniela Isabel Brayer Pereira,[4] Luís Antonio Sangioni,[1] Águeda Castagna Vargas,[1] and Sônia de Avila Botton[1,*]

Abstract

Nontyphoidal *Salmonella* are one of the leading causes of foodborne diseases in the world. As poultry products are recognized as main sources of human salmonellosis, nontyphoidal *Salmonella* control has become a global issue for the poultry industry. The increasing antimicrobial resistance in poultry-related nontyphoidal *Salmonella* serovars is a global matter of concern. By monitoring the evolution of antimicrobial resistance, alternative treatments can be identified and possible restrictions in the treatment of systemic human salmonellosis foreseen. A meta-analysis was conducted to assess the profile and temporal evolution of the antimicrobial resistance of nontyphoidal *Salmonella* of poultry and human origin in Brazil, isolated in the period from 1995 to 2014. Four databases were researched; twenty-nine articles met the eligibility criteria and were included in the meta-analysis. In the nontyphoidal isolates of poultry origin, the highest levels of antimicrobial resistance were verified for sulfonamides (44.3%), nalidixic acid (42.5%), and tetracycline (35.5%). In the human-origin isolates, the resistance occurred mainly for sulfonamides (46.4%), tetracycline (36.9%), and ampicillin (23.6%). Twenty-two articles described results of antimicrobial resistance specifically for *Salmonella* Enteritidis, also enabling the individual meta-analysis of this serovar. For most antimicrobials, the resistance levels of *Salmonella* Enteritidis were lower than those found when considering all the nontyphoidal serovars. In the poultry-origin isolates, a quadratic temporal distribution was observed, with reduced resistance to streptomycin in *Salmonella* Enteritidis and in all nontyphoidal serovars, and a linear increase of resistance to nalidixic acid in *Salmonella* Enteritidis. In the human-origin isolates, a linear increase was identified in the resistance to nalidixic acid in *Salmonella* Enteritidis and in all the nontyphoidal isolates, and to gentamicin in *Salmonella* Enteritidis. Continuous monitoring of the development and spread of antimicrobial resistance could support the measurement of the consequences on poultry and human health.

Keywords: antimicrobial resistance, *Salmonella,* human, poultry, meta-analysis, systematic review

Introduction

Salmonella is an enterobacteria found in several species of animals and one of the main microorganisms transmitted by food worldwide. It is responsible for the second most reported zoonosis in humans in Europe (EFSA, 2015b) and the most reported in the United States (CDC, 2014). *Salmonella enterica* subspecies *enterica* is the most frequently involved and comprises >1500 serovars (EFSA, 2015b). More broadly speaking, the *Salmonella* genus can be split into two large groups: typhoidal, which includes the host-restricted serovars, and the nontyphoidal, comprising the other host ubiquitous serovars (Hur *et al.*, 2012). Infection by nontyphoidal *Salmonella* can present different clinical manifestations in humans. Gastroenteritis is the most common manifestation and most cases are self-limiting (Jasson

[1]Programa de Pós-Graduação em Medicina Veterinária (PPGMV), Departamento de Medicina Veterinária Preventiva, Centro de Ciências Rurais (CCR), Universidade Federal de Santa Maria (UFSM), Santa Maria, Brazil.
[2]Laboratório de Sanidade e Genética Animal, Embrapa Suínos e Aves, Concordia, Brazil.
[3]Departamento de Zootecnia, CCR, UFSM, Santa Maria, Brazil.
[4]Departamento de Microbiologia e Parasitologia, Instituto de Biologia (IB), Universidade Federal de Pelotas (UFPel), Pelotas, Brazil.
*Adviser.

and Butaye, 2012). However, in immunocompromised elderly and child patients, or in cases of serious systemic infection, antimicrobial treatment is essential. In this context, broad-spectrum fluoroquinolones and cephalosporins are fundamental for the treatment of salmonellosis, and the emergence of antimicrobial resistance directly affects treatment evaluation (Ruiz *et al.*, 2004; EFSA, 2015a).

Antimicrobials have been key in controlling bacterial infections; however, over the course of time, use of these drugs has promoted the selection of resistant bacteria. The choice of unsuitable antimicrobials, dosage, and treatment times has fomented the emergence of resistant strains. The spread of resistant microorganisms has been supported by inefficient infection control measures, improper sanitary conditions, and inappropriate manipulation of foodstuffs (WHO, 2015). Contaminated poultry products are considered important sources of human *Salmonella* infection. As a rule, poultry colonized by nontyphoidal *Salmonella* do not develop clinical signs of illness and do not require antimicrobial treatment, but they do offer fertile conditions for the bacteria to disseminate in the flock (EFSA, 2015a). In this condition, the bacteria remain under the selective pressure of any other antimicrobial that is administered.

Brazil is the world's leading exporter of chicken meat, and a portion of its table eggs production is sold to foreign markets (ABPA, 2015), this is why the impact of antimicrobial resistance could have international reach. Therefore, epidemiological surveillance of microorganisms resistant to antimicrobials is essential to gather information about the magnitude and trends of such resistance, allowing for alternative treatments to be planned and identified and the possible limitations in the treatment of severe cases of salmonellosis to be foreseen. Several studies about antimicrobial resistance in *Salmonella* have been independently accomplished in Brazil, representing different geographic areas and periods of time (Wilson, 2004; Oliveira *et al.*, 2005, 2012; Vaz *et al.*, 2010; Campioni *et al.*, 2012, 2014; Voss-Rech *et al.*, 2015). Consequently, the results obtained in each study are variable and hinder an all-encompassing interpretation. In such cases, meta-analysis represents an important tool to support retrospective studies into antimicrobial resistance. Integrated analysis of data from previous studies can allow one to adopt a more realistic perspective of the antimicrobial resistance of *Salmonella* spp. and its temporal evolution in Brazil. This information is important for establishing strategies to prevent the emergence and spreading of resistant strains in the country. We, therefore, carried out a systematic review and meta-analysis study with the aim of evaluating the profile and temporal evolution of the antimicrobial resistance of nontyphoidal *Salmonella* from humans and poultry over the past 20 years in Brazil.

Materials and Methods

Search strategy

To obtain the data to be included in the research, an extensive systematic review of the literature was performed to identify the studies that have assessed the antimicrobial resistance of *Salmonella* spp., published between January 1995 and May 2015. This review was conducted in four stages: identification, screening, eligibility, and inclusion, as recommended in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses—PRISMA (Moher *et al.*, 2009). The following online databases were consulted: Web of Science, PubMed, SciELO, and Science Direct. The keywords searched for in the articles included ("antimicrobial resistance" or "microbial resistance" or "bacterial resistance" or "resistance pattern" or resistance or susceptibility) and *Salmonella* and Brazil or Brasil.

Study selection

The initial selection prioritized the identification of articles related to the study scope, based on the title and abstract. All selected articles were read in full for a second selection stage, in accordance with the following eligibility criteria: (1) isolated in Brazil, (2) isolated from poultry and/or human origin, (3) isolated between 1995 and 2014, (4) number of isolates and year of isolation identified, (5) results separated by origin, (6) not directed at multiresistance, and (7) employed the disk diffusion susceptibility test. Furthermore, the references cited in the selected articles were also analyzed and included in the study, when pertinent. The information extracted from the articles was systematized in a spreadsheet (Microsoft Excel, 2010). Studies that present data from different years separately were included in the database as different observations. For articles in which the results of multiple years were presented together, the average year of the period was considered.

Quality assessment

The studies included in the database were categorized by quality, in accordance with the following set of criteria: informed the criterion for interpreting zones of inhibition, used a standard strain for quality control of the tests, employed the international methodology approved by the Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST), and evaluated >20 nontyphoidal *Salmonella* isolates. The articles received a score from 0 to 4 according to the number of criteria met.

Statistical analysis

The data were analyzed using the statistical software SAS, version 9.4. The antimicrobial resistance of the nontyphoidal *Salmonella* isolates was assessed in function of the percentage of resistance by serovar and the origin. Resistance against the most commonly used antimicrobials was compared, for both humans and poultry. The comparisons were made by means of the Kruskal–Wallis test and, when any differences were found between antimicrobials, the Bonferroni test was applied to compare the mean averages.

Regression analysis was conducted to assess the temporal evolution of the antimicrobials, in which the choice of the models was based on the significance of the linear, quadratic, and cubic coefficients, using the Student's *t*-test at 5% probability. This analysis only considered those antimicrobials that were assessed for at least five different years. For regression analysis, square root transformation was applied to the antimicrobials that failed to present normality. The temporal distributions graphs of the antimicrobial resistance were generated by plotting the level of resistance of each observation.

To classify the levels of antimicrobial resistance, the following parameters were used: rare, <0.1%; very low, 0.1% to 1%; low, >1% to 10%; moderate, >10% to 20%; high, >20% to 50%; very high, >50% to 70%; extremely high, >70% (EFSA, 2015b).

Results

Systematic review

The systematic review of the literature process is presented in Figure 1. Initially, 473 articles were identified, of which 29 met all the eligibility criteria and were included in the meta-analysis. Of those, 17 articles assessed nontyphoidal *Salmonella* isolated from poultry, 8 from humans, and 4 from both humans and poultry (Table 1).

Quality assessment

Of those articles included in the meta-analysis, 22 articles (75.8%) met two or more pre-established criteria and were considered of higher quality. Only 4 articles clearly mentioned the criteria for interpreting zones of inhibition, 14 articles described the use of strains for quality control of the antimicrobial susceptibility tests, 24 articles used and/or informed the international methodology (CLSI or EUCAST), and 23 articles tested >20 isolates. All the categorized articles were analyzed, irrespective of the score achieved.

Antimicrobial resistance

In the 29 articles included in the study, a total of 2119 nontyphoidal *Salmonella* isolates were assessed, 1272 of which were recovered from poultry (drag swab, cloacal swab, viscera, stools, meconium, carcasses, chicken portions, feed, broiler litter, and table eggs) and 847 recovered from humans (stools, blood, and other fluids). Of these, 22 articles showed positive results for *Salmonella* Enteritidis, enabling individual analysis of this serovar.

Forty-five different antimicrobials were tested for at least one of the studies included in the database. The most frequently tested antimicrobials were selected for meta-analysis: ampicillin, cefalotin, chloramphenicol, ciprofloxacin, enrofloxacin, gentamicin, nalidixic acid, norfloxacin, streptomycin, sulfonamides, trimethoprim/sulfamethoxazole, and tetracycline. Furthermore, cefotaxime and ceftriaxone were also included, because of their clinical importance. Analysis of these 14 antimicrobials comprised 556 observations. Observations corresponded to the frequency (%) of antimicrobial resistance found in each given article according to the source (poultry or human).

Antimicrobial resistance in human isolates

In the nontyphoidal *Salmonella* isolates from human, the highest resistance levels were found against sulfonamides (46.4%), tetracycline (28%), and ampicillin (24.5%). For *Salmonella* Enteritidis, the highest resistance levels were

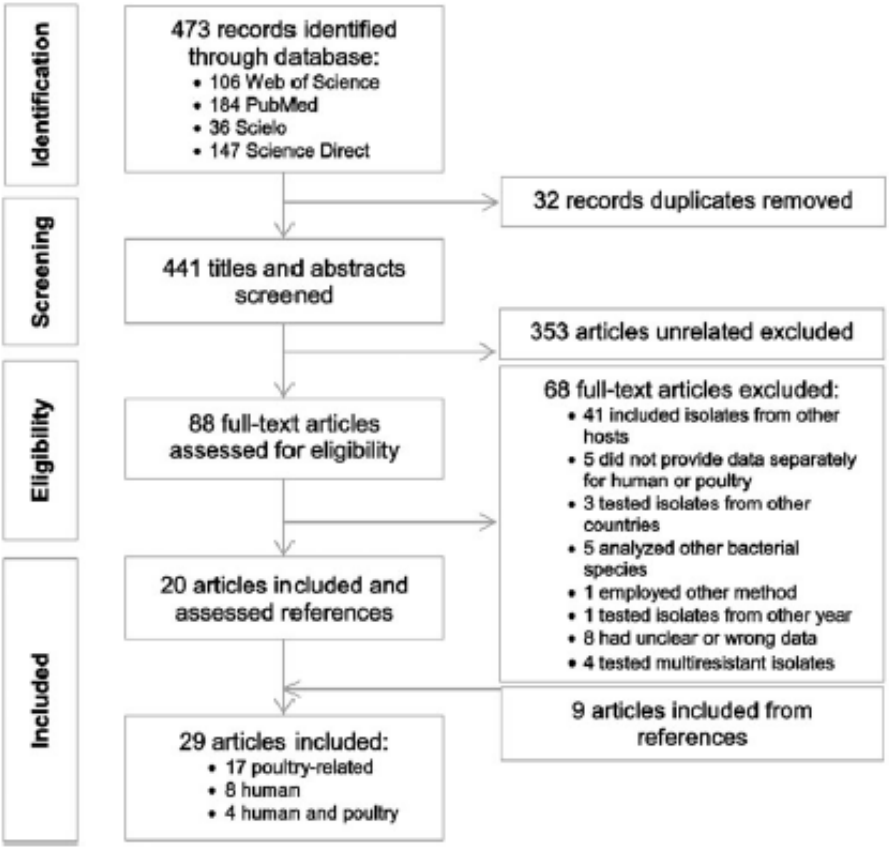


FIG. 1. Flow diagram of articles selection.

TABLE 1. CHARACTERISTICS OF THE SELECTED ARTICLES

Reference	*Region of Brazil*	*Isolation period*	*Average year*	*Origin of isolate*	*No. of* Salmonella *Enteritidis*	*No. of other serovars*	*Total no. of isolates*
Peresi *et al.* (1998)	Southeast	1993–1997	1995	Poultry	5	0	5
			1995	Human	18	0	18
Santos *et al.* (2000)	Southeast	1996–1997	1997	Poultry	29	19	48
Oplustil *et al.* (2001)	South, Southeast, Mid-West, Northeast	1998–1999	1999	Human	0	57	57
Baú *et al.* (2001)	South	1997–1998	1998	Poultry	10	3	13
Castro *et al.* (2002)	Southeast	1985–1999	1994	Human	128	0	128
Delicato *et al.* (2004)	South	1999–2000	2000	Human	14	7	21
Wilson (2004)	NI	1998–2000	1999	Poultry	15	11	26
Oliveira *et al.* (2005)	South	1995–1996	1996	Human	17	0	17
			1996	Poultry	43	0	43
Cortez *et al.* (2006)	Southeast	2003–2004	2004	Poultry	0	29	29
Cardoso *et al.* (2006)	South	1995–1996	1996	Poultry	80	0	80
Fonseca *et al.* (2006)	Southeast	1996–2001	1999	Human	0	35	35
Pereira *et al.* (2007)	NI	1999–2003	2001	Human	0	10	10
Ribeiro *et al.* (2007)	South	1996	1996	Poultry	21	4	25
Ribeiro *et al.* (2008)	South	1999	1999	Poultry	32	0	32
		2000	2000	Poultry	28	0	28
		2001	2001	Poultry	19	0	19
Lima *et al.* (2009)	NI	1994–2006	2000	Poultry	153	67	220
Duarte *et al.* (2009)	Northeast	2004	2004	Poultry	5	14	19
Vaz *et al.* (2010)	South	1995–2003	1999	Poultry	53	0	53
		1995–1996	1996	Poultry	9	0	9
		1995–1996	1996	Human	14	0	14
Kottwitz *et al.* (2011)	NI	2002–2006	2004	Poultry	7	0	7
		2002–2006	2004	Human	4	0	4
Medeiros *et al.* (2011)	South, Southeast, Mid-West, North, Northeast	2004–2006	2005	Poultry	122	128	250
Reis *et al.* (2011)	NI	1990–1999	1995	Human	0	70	70
		2000–2008	2004	Human	0	327	327
Campioni *et al.* (2012)	Southeast	1992–1995	1994	Human	11	0	11
		1996–2000	1998	Human	22	0	22
		2001–2005	2003	Human	21	0	21
		2006–2010	2008	Human	12	0	12
Oliveira *et al.* (2012)	South	1999–2006	2003	Human	80	0	80
Kottwitz *et al.* (2012)	South	2002–2006	2004	Poultry	38	0	38
Kottwitz *et al.* (2013)	South	2003–2006	2005	Poultry	22	4	26
Scur *et al.* (2014)	South	2006–2010	2008	Poultry	16	102	118
Campioni *et al.* (2014)	South, Southeast, Mid-West, Northeast	2004–2010	2007	Poultry	60	0	60
Albuquerque *et al.* (2014)	Northeast	2013–2014	2014	Poultry	3	0	3
Pandini *et al.* (2014)	South	2010–2011	2011	Poultry	0	39	39
Voss-Rech *et al.* (2015)	South	2010–2011	2011	Poultry	0	82	82

NI, not informed.

against nalidixic acid (21.1%), ampicillin (13%), and tetracycline (9.4%) (Table 2).

It was found that the nontyphoidal serovars of human origin displayed a growing linear temporal evolution of the antimicrobial resistance against nalidixic acid ($p=0.0004$, $R^2=80.5\%$) (Fig. 2A). For *Salmonella* Enteritidis, resistance against nalidixic acid ($p=0.0005$, $R^2=88.5\%$) and gentamicin ($p=0.0005$, $R^2=88.5\%$) also increased over the course of the years (Fig. 2B, C). No temporal effect was identified for any of the other antimicrobials.

Antimicrobial resistance in poultry isolates

In the nontyphoidal *Salmonella* isolated from poultry, the highest levels of resistance were found for sulfonamides (44.3%), nalidixic acid (42.5%), and tetracycline (35.6%).

TABLE 2. AVERAGE ANTIMICROBIAL RESISTANCE OF NONTYPHOIDAL *SALMONELLA* AND *SALMONELLA* ENTERITIDIS ISOLATED FROM POULTRY AND HUMANS FROM 1995 TO 2014

		Poultry, % (n)		*Human*, % (n)	
Class	*Antimicrobial*	Salmonella *Enteritidis*	*All*	Salmonella *Enteritidis*	*All*
Aminoglycosides	Streptomycin	20.3 (564)	22.5 (1000)	3.3 (177)	12.4 (609)
	Gentamicin	6.7 (643)	6.6 (1141)	3.8 (271)	13.3 (323)
Cephems	Ceftriaxone	NA	NA	0 (80)	11.2 (189)
	Cefotaxime	0 (82)	12.1 (132)	NA	NA
	Cefalotin	15.5 (275)	24.2 (592)	2.1 (243)	12.6 (295)
Phenicols	Chloramphenicol	1.3 (620)	2.9 (1026)	1.8 (327)	15.2 (833)
Folate pathway inhibitors	Sulfonamide	43.8 (478)	44.3 (684)	NA	46.4 (428)
	Trimethoprim/ sulfamethoxazole	2.8 (459)	8.2 (879)	6.2 (324)	14.8 (398)
Penicillin	Ampicillin	9 (628)	14.8 (1048)	13 (327)	24.5 (833)
Quinolones	Nalidixic acid	48.2 (631)	42.5 (989)	21.1 (305)	18.9 (350)
	Ciprofloxacin	1.4 (609)	1.4 (1060)	0.7 (133)	0.5 (242)
	Enrofloxacin	9.6 (479)	7.3 (876)	NA	NA
	Norfloxacin	0.8 (254)	1.8 (498)	NA	NA
Tetracycline	Tetracycline	32 (638)	35.6 (1136)	9.4 (305)	28 (747)

All, nontyphoidal serovars, including *Salmonella* Enteritidis; NA, not analyzed (less than four articles have tested these antimicrobials).

For *Salmonella* Enteritidis, the antimicrobial resistance was more prominent against nalidixic acid (48.2%), sulfonamides (43.8%), and tetracycline (32%) (Table 2).

The antimicrobial resistance of the poultry nontyphoidal *Salmonella* serovars and *Salmonella* Enteritidis against streptomycin showed a quadratic temporal distribution ($p=0.029$, $R^2=33.9\%$) (Fig. 3A) and ($p=0.029$, $R^2=33.9\%$) (Fig. 3B), respectively. Furthermore, *Salmonella* Enteritidis displayed a growing linear temporal evolution against nalidixic acid ($p=0.015$, $R^2=35.3\%$) (Fig. 3C). No temporal effect was identified for any of the other antimicrobials. In general, *Salmonella* Enteritidis in isolation showed lower levels of antimicrobial resistance than all the nontyphoidal serovars ($p=0.036$).

Discussion

In the isolates recovered from poultry, the highest levels of antimicrobial resistance were against sulfonamides, nalidixic acid, and tetracycline, respectively. For the human isolates, sulfonamides, tetracycline, and ampicillin showed the highest levels of resistance (Table 2). Similar results have also been found in other countries (Jasson and Butaye, 2012; Van *et al.*, 2012; CDC, 2015; EFSA, 2015a). These antimicrobial agents are among the oldest groups used in the treatment of bacterial infections, both in human and in veterinary medicine. The tetracyclines and sulfonamides were used as additives in animal feeds in Brazil until 1998, when their use was restricted to therapeutic purposes. However, these drugs still exert selection pressure on the microorganisms. Despite the high levels of resistance found, these antimicrobials are not among those considered critical for the treatment of human salmonellosis (WHO, 2011).

The resistance to nalidixic acid of the poultry isolates and the *Salmonella* Enteritidis isolates from humans was high (>20% to 50%) (Table 2) in the analyzed period; the resistance levels increased significantly over the course of time, for nontyphoidal *Salmonella* isolated both from humans and from poultry (Figs. 2A, B and 3C). The increased resistance

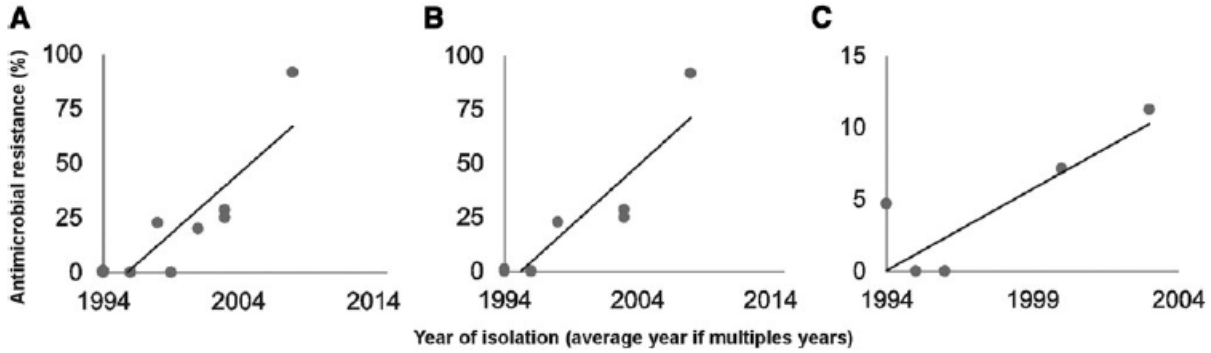


FIG. 2. Temporal evolution of the antimicrobial resistance of *Salmonella* of human origin. **(A)** Resistance of nontyphoidal *Salmonella* to nalidixic acid (*n*: 350; *a*: 7). **(B)** Resistance of *Salmonella* Enteritidis to nalidixic acid (*n*: 305; *a*: 5). **(C)** Resistance of *Salmonella* Enteritidis to gentamicin (*n*: 271; *a*: 6). *n*, number of isolates; *a*, number of articles.

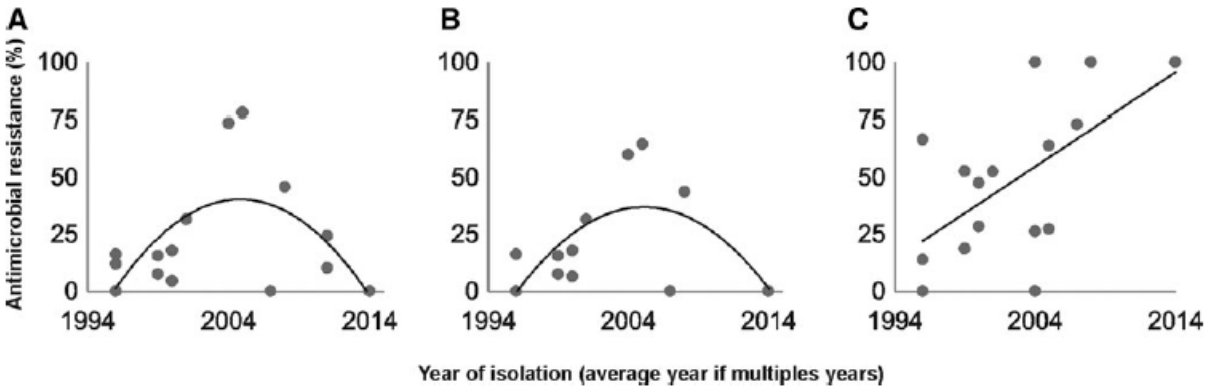


FIG. 3. Temporal evolution of the antimicrobial resistance of *Salmonella* of poultry origin. **(A)** Resistance of nontyphoidal *Salmonella* to streptomycin (*n*: 1000; *a*: 11). **(B)** Resistance of *Salmonella* Enteritidis to streptomycin (*n*: 564; *a*: 9). **(C)** Resistance of *Salmonella* Enteritidis to nalidixic acid (*n*: 631; *a*: 11). *n*, number of isolates; *a*, number of articles.

to quinolones has also been found in nontyphoidal *Salmonella* of human origin in the United States since 1996 (Stevenson *et al.*, 2007; WHO, 2015) and in China between 2009 and 2012 (Lai *et al.*, 2014). Isolates from poultry displayed an increase in resistance to nalidixic acid in Austria, Czech Republic, Germany, Poland, and Slovakia (EFSA, 2015a). For some antimicrobials, differences in the occurrence of resistance are observed between countries. However, this increase has been attributed to the broad use of these antimicrobials in both human and veterinary medicine (Angulo *et al.*, 2004; Stevenson *et al.*, 2007).

Despite nalidixic acid not being one of the critical antimicrobials in human treatment, the resistance of this drug in Enterobacteriaceae is generally correlated to reduced susceptibility to ciprofloxacin (Lai *et al.*, 2014). Ciprofloxacin, a second-generation quinolone (fluoroquinolone), is the medication of choice for treating serious cases of human salmonellosis. Treatments with fluoroquinolones have failed in patients infected with *Salmonella* spp. resistant to nalidixic acid (Dimitrov *et al.*, 2007; Stevenson *et al.*, 2007). This resistance is primarily attributed to point mutations in the gene regions where resistance to quinolones is determined, including the gyrase and topoisomerase IV genes (Cavaco *et al.*, 2009; EFSA, 2015a), as well as by active efflux mechanisms (Hur *et al.*, 2012). In this study, we found low resistance levels to ciprofloxacin, <1.5%, regardless of the origin of the isolates (Table 2). Low resistance levels to quinolones have been found in nontyphoidal *Salmonella* of human origin in the United States (3.5%) (CDC, 2015) and the European Union (EU) (3.8%) (EFSA, 2015a). However, in the EU, highest levels of microbiological resistance were found in isolates from chickens (53.8%), although lower levels (<10.0%) were recorded in Denmark, France, Ireland, and the United Kingdom.

The second most important group for treating human salmonellosis is the third-generation cephalosporins, especially for serious infections in children, in whom the use of fluoroquinolones can cause side effects (EFSA, 2015a). In the antimicrobials assessed in this work, this group is represented by ceftriaxone and cefotaxime. Resistance to these antimicrobials results from the presence of genes, usually found on plasmids, encoding extended-spectrum β-lactamase enzymes or AmpC enzymes, which can degrade the chemical structure of the antimicrobial (Miriagou *et al.*, 2004). Our results demonstrated that resistance to cefotaxime and to ceftriaxone was moderate (>10% to 20%) in the nontyphoidal isolates and rare (<0.1%) in *Salmonella* Enteritidis, regardless of the origin (Table 2). Lower levels of antimicrobial resistance in the nontyphoidal *Salmonella* have been described in the United States and the EU. In the United States, 2.5% of resistance was related to ceftriaxone in human isolates (CDC, 2015), and in the EU, this level was 1.4% and 3.2% to cefotaxime in human and poultry, respectively (EFSA, 2015a).

A temporal evolution of the antimicrobial resistance was also observed for streptomycin and gentamicin, belonging to the class of aminoglycosides. Resistance to members of this class is associated with enzymes production, changes in uptake and efflux, action of membrane proteases, and target modification (Becker and Cooper, 2013). The *Salmonella* Enteritidis isolates of human origin displayed increased resistance to gentamicin until 2003, the last year of assessment in the articles (Fig. 2C). This antimicrobial is widely used in human treatment, which may have promoted the selection of resistant isolates. For streptomycin, the isolates of nontyphoidal *Salmonella* and *Salmonella* Enteritidis of poultry origin presented a quadratic distribution effect of antimicrobial resistance. We observed an increase in resistance until 2005, followed by a sharp decline until the end of the studied period (Fig. 3A, B). Streptomycin is commonly used in treating infectious diseases in animals, it is a low-cost drug, discovered more than 70 years ago, which may have contributed toward the increased resistance in the first decade of assessment. The decline observed in recent years has also been demonstrated in other countries (CDC, 2015) and could have resulted from reduced use of streptomycin in poultry.

In Brazil, *Salmonella* Enteriditis was the most frequently reported serovar in the poultry industry for more than two decades. Indeed, the majority of the studies reviewed in this study presented specific data about *Salmonella* Enteriditis (Table 1). A national program for vaccination and control was implemented in breeders (Brazil, 2003), as well as the establishment of continuous monitoring of broiler chicken and turkey flocks at farm level (Brazil, 2009), and, recently, a decline has been reported in this serovar in broiler chickens (Pandini *et al.*, 2014; Voss-Rech *et al.*, 2015). However, *Salmonella* Enteritidis is among the serovars most commonly

involved in human infections in Brazil (Baú *et al.*, 2001; Delicato *et al.*, 2004; Capalonga *et al.*, 2014) and also in other countries (CDC, 2014; EFSA, 2015b). Therefore, monitoring the antimicrobial resistance of *Salmonella* Enteritidis is indispensable. In this study, *Salmonella* Enteritidis, when individually analyzed, was more susceptible to the majority of the antimicrobials than all the nontyphoidal serovars (Table 2). In contrast to its prevalence in human infections, *Salmonella* Enteritidis is reported as a more susceptible serovar to antimicrobials (Hur *et al.*, 2012; Van *et al.*, 2012; EFSA, 2015a).

Study limitation

Several studies were excluded from the meta-analysis because of inconsistencies in the presentation of the results, conflicting information in different sections of the article, or because they failed to separate the results by origin, and others (Fig. 1). Despite this critical evaluation, some limitation could not be overcome in this study: (1) it was not possible to standardize interpretation of the results, because of modifications to the diameter ranges of the zones of inhibition used over the years or because of an absence of information of the interpretation criteria adopted; (2) the number of isolates from each geographic region varied over the years, tending to the greater representation of some regions at certain periods of time; and (3) in the articles that presented the results of multiple years together, the average year of the period was considered in the temporal evaluation.

Similar difficulties were observed by Moodley *et al.* (2014), and we can corroborate their recommendation that, for new meta-analysis studies, it would be important for the resistance interpretation criteria to be harmonized, as well as the presentation of standardized results.

Conclusions

The results demonstrated that the highest resistance levels were found for the oldest antimicrobials. The resistance to the majority of the antimicrobials was not changed over the course of time, except for streptomycin, nalidixic acid, and gentamicin, the observed effect of which varied in accordance with the origin or serovar of the isolates. In addition, *Salmonella* Enteritidis displayed lower levels of antimicrobial resistance than all the nontyphoidal serovars. These results reinforce the importance of epidemiological surveillance and the need to limit the continued evolution of antimicrobial resistance to preserve the action of available drugs.

Disclosure Statement

No competing financial interests exist.

References

[ABPA] Associação Brasileira de Proteína Animal. Relatório Anual de Atividades 2014. São Paulo, 2015. Available at: http://abpa-br.com.br/files/publicacoes/c59411a243d6dab1da8e605be58348ac.pdf Accessed June 15, 2016.

Albuquerque AH, Maciel WC, Lopes ED, Teixeira RSD, Salles RPR, Machado DN, Bezerra WGA, Vasconcelos RH, Mendonça SV, Carbó CB. Presence of *Salmonella* spp. in one-day-old chicks from hatcheries in the metropolitan region of Fortaleza, Brazil. Acta Sci Vet 2014;42:1222.

Angulo F, Nargund V, Chiller T. Evidence of association between use of antimicrobial agents in food animals and antimicrobial resistance among bacteria isolated from humans and the human health consequences of such resistance. J Vet Med 2004;51:374–379.

Baú AC, Carvalhal JB, Aleixo JAG. Prevalência de *Salmonella* em produtos de frangos e ovos de galinha comercializados em Pelotas, RS, Brasil. Cienc Rural 2001;31:303–307.

Becker B, Cooper MA. Aminoglycoside antibiotics in the 21st century. ACS Chem Biol 2013;8:105–115.

Brazil. Ministry of Agriculture, Livestock and Food Supply. Normative Instruction SDA no. 78, of 3 November 2003. Normas técnicas para controle e certificação de núcleos e estabelecimentos avícolas como livres de *S*. Gallinarum e de *S*. Pullorum e livres ou controlados para *S*. Enteritidis e para *S*. Typhimurium. Diário Oficial da União 2003;1:3.

Brazil. Ministry of Agriculture, Livestock and Food Supply. Joint Official Communication DSA/DIPOA no. 001/2009 of 15 January 2009. Procedimentos para monitoramento de estabelecimentos de frango de corte e perus para Salmoneloses aviarias. Departamento de Saude Animal 2009;1–4.

Campioni F, Moratto Bergamini AM, Falcão JP. Genetic diversity, virulence genes and antimicrobial resistance of *Salmonella* Enteritidis isolated from food and humans over a 24-year period in Brazil. Food Microbiol 2012;32:254–264.

Campioni F, Zoldan MM, Falcao JP. Characterization of *Salmonella* Enteritidis strains isolated from poultry and farm environments in Brazil. Epidemiol Infect 2014;142:1403–1410.

Capalonga R, Ramos RC, Both JM, Soeiro ML, Longaray SM, Haas S, Tondo EC. *Salmonella* serotypes, resistance patterns, and food vehicles of salmonellosis in southern Brazil between 2007 and 2012. J Infect Dev Ctries 2014;8:811–817.

Cardoso MO, Ribeiro AR, dos Santos LR, Pilotto F, de Moraes HLS, Salle CTP, Rocha SLS, do Nascimento WPD. Antibiotic resistance in *Salmonella* Enteritidis isolated from broiler carcasses. Braz J Microbiol 2006;37:368–371.

Castro FA, Santos VR, Martins CHG, Fernandes SA, Zaia JE, Martinez R. Prevalence and antimicrobial susceptibility of *Salmonella* serotypes in patients from Ribeirão Preto, São Paulo, Brazil, between 1985 and 1999. Braz J Infect Dis 2002;6:244–251.

Cavaco LM, Hasman H, Xia S, Aarestrup FM. qnrD, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* serovar Kentucky and Bovismorbificans strains of human origin. Antimicrob Agents Chemother 2009;53:603–608.

[CDC] Centers for Disease Control and Prevention. *Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet Surveillance Report for 2012 (Final Report)*. Atlanta, Georgia: U.S. Department of Health and Human Services, CDC, 2014.

[CDC] Centers for Disease Control and Prevention. *National Antimicrobial Resistance Monitoring System (NARMS): Human Isolates Final Report, 2013*. Atlanta, Georgia: U.S. Department of Health and Human Services, CDC, 2015.

Cortez A, Carvalho A, Ikuno A, Bürger K, Vidal-Martins A. Resistência antimicrobiana de cepas de *Salmonella* spp. isoladas de abatedouros de aves. Arq Inst Biol 2006;73:157–163.

Delicato ER, Mikcha JMG, Fernandes SA, Pelayo JS. Resistance profile to antimicrobials of *Salmonella* spp. isolated from human infections. Braz Arch Biol Technol 2004;47:193–197.

Dimitrov T, Udo EE, Albaksami O, Kilani AA, Shehab DM. Ciprofloxacin treatment failure in a case of typhoid fever caused by *Salmonella enterica* serotype Paratyphi A with reduced susceptibility to ciprofloxacin. J Med Microbiol 2007;56:277–279.

Duarte DAM, Ribeiro AR, Vasconcelos AMM, Santos SB, Silva JVD, Andrade PL, Falcão LSA. Occurrence of *Salmonella* spp. in broiler chicken carcasses and their susceptibility to antimicrobial agents. Braz J Microbiol 2009;40: 569–573.

[EFSA] European Food Safety Authority and [ECDC] European Centre for Disease Prevention and Control. EU Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. EFSA J 2015a;13:4036, 178 pp.

[EFSA] European Food Safety Authority and [ECDC] European Centre for Disease Prevention and Control. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA J 2015b;13:4329, 191 pp.

Fonseca EL, Mykytczuk OL, Asensi MD, Reis EMF, Ferraz LR, Paula FL, Ng LK, Rodrigues DP. Clonality and antimicrobial resistance gene profiles of multidrug-resistant *Salmonella enterica* serovar Infantis isolates from four public hospitals in Rio de Janeiro, Brazil. J Clin Microbiol 2006; 44:2767–2772.

Hur JC, Jawale C, Lee JE. Antimicrobial resistance of *Salmonella* isolated from food animals: A review. Food Res Int 2012;45:819–830.

Jasson V, Butaye P. Report on susceptibility of *Salmonella* serotypes in Belgium. CODA-CERVA (Centrum voor Onderzoek in Diergeneeskunde en Agrochemie-Centre d'Etudeet des Recherches Vétérinaires et Agrochimiques). Federal Public Service Health, Food Chain Security and Environment. Belgium. 2012. Available at: www.amcra.be/sites/default/files/bestanden/Report%20Salmonella%20data%202012.pdf Accessed May 13, 2016.

Kottwitz LB, Leão JA, Back A, Rodrigues DP, Magnani M, Oliveira TC. Commercially laid eggs vs. discarded hatching eggs: Contamination by *Salmonella* spp. Braz J Microbiol 2013;44:367–370.

Kottwitz LBM, Scheffer MC, Dalla-Costa LM, Farah S, Moscalewski WSB, Magnani M, de Oliveira TCRM. Molecular characterization and resistance profile of *Salmonella* Enteritidis PT4 and PT9 strains isolated in Brazil. J Med Microbiol 2011;60:1026–1031.

Kottwitz LBM, Scheffer MC, Dalla Costa LM, Leitão JA, Back A, Rodrigues DP, Magnani M, de Oliveira TCRM. Perfil de resistência a antimicrobianos, fagotipagem e caracterização molecular de cepas de *Salmonella* Enteritidis de origem avícola. Semina: Ciências Agrárias 2012;33: 705–712.

Lai J, Wu C, Wu C, Qi J, Wang Y, Wang H, Liu Y, Shen J. Serotype distribution and antibiotic resistance of *Salmonella* in food-producing animals in Shandong province of China, 2009 and 2012. Int J Food Microbiol 2014;180: 30–38.

Lima ET, Andreatti Filho RL, Pinto JPAN. Perfil de susceptibilidade antimicrobiana de sorotipos de *Salmonella* isolados de produtos avícolas. Vet Zootec 2009;16:394–400.

Medeiros MAN, Oliveira DCN, Rodrigues DPR, Freitas DRC. Prevalence and antimicrobial resistance of *Salmonella* in chicken carcasses at retail in 15 Brazilian cities. Rev Panam Salud Publica 2011;30:555–560.

Miriagou V, Tassios PT, Legakis NJ, Tzouvelekis LS. Expanded-spectrum cephalosporin resistance in non-typhoid *Salmonella*. Int J Antimicrob Agents 2004;23:547–555.

Moher D, Liberati A, Tetzlaff J, Altman DG; The PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. PLoS Med 2009;6: e1000097.

Moodley A, Damborg P, Nielsen SS. Antimicrobial resistance in methicillin susceptible and methicillin resistant *Staphylococcus pseudintermedius* of canine origin: Literature review from 1980 to 2013. Vet Microbiol 2014;171: 337–341.

Oliveira FA, Pasqualotto AP, Silva WP, Tondo EC. Characterization of *Salmonella* Enteritidis isolated from human samples. Food Res Int 2012;45:1000–1003.

Oliveira SD, Siqueira Flores F, dos Santos LR, Brandelli A. Antimicrobial resistance in *Salmonella* Enteritidis strains isolated from broiler carcasses, food, human and poultry-related samples. Int J Food Microbiol 2005;97:297–305.

Oplustil CP, Nunes R, Mendes C; RESISTNET Group. Multicenter evaluation of resistance patterns of *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella* spp and *Shigella* spp isolated from clinical specimens in Brazil: RESISTNET surveillance program. Braz J Infect Dis 2001;5:8–12.

Pandini JA, da Silva Pinto FG, Muller JM, Weber LD, Moura AC. Ocorrência e perfil de resistencia antimicrobiana de sorotipos de *Salmonella* spp. isolados de aviários do Paraná, Brasil. Arq Inst Biol 2014. Available at: http://www.scielo.br/pdf/aib/v82/1808-1657-aib-1808-1657000352013.pdf Accessed May 13, 2016.

Pereira CS, Medeiros LM, Costa RG, Festivo ML, dos Reis EMF, Seki LM, Rodrigues DP. Phage typing and multidrug resistance profile in *S.* Typhimurium isolated from different sources in Brazil from 1999 to 2004. Braz J Microbiol 2007; 38:385–390.

Peresi JTMP, Almeida IAZC, Lima SI, Marques DF, Rodrigues ECA, Fernandes SA, Gelli DS, Irino K. Surtos de enfermidades transmitidas por alimentos causados por *Salmonella* Enteritidis. Rev Saúde Pública 1998;32:477–483.

Reis EMF, Rodrigues DP, Freitas-Almeida AC, Hofer E. Prevalence of R-type ACSSuT in strains of *Salmonella* serovar Typhimurium DT193 isolated from human infections in Brazil. Rev Panam Salud Publica 2011;29:387–392.

Ribeiro AR, Kellermann A, Santos LR, Bessa MC, Nascimento VP. *Salmonella* spp. in raw broiler parts: Occurrence, antimicrobial resistance profile and phage typing of the *Salmonella* Enteritidis isolates. Braz J Microbiol 2007;38:296–299.

Ribeiro AR, Kellermann A, Santos LR, Nascimento VP. Antimicrobial resistance in *Salmonella* Enteritidis isolated from clinical and environmental broiler chickens and breeders broiler. Arq Bras Med Vet Zootec 2008;60:1259–1262.

Ruiz M, Rodriguez JC, Escribano I, Royo G. Available options in the management of non-typhi *Salmonella*. Expert Opin Pharmacother 2004;5:1737–1743.

Santos DMS, Berchieri JA, Fernandes SA, Tavechio AT, Amaral LA. *Salmonella* em carcaças de frango congeladas. Pesq Vet Bras 2000;20:39–42.

Scur MC, Pinto FGS, De Bona EAM, Weber LD, Alves LFA, Moura AC. Occurrence and antimicrobial resistance of *Salmonella* serotypes isolates recovered from poultry of Western Paraná, Brazil. Afr J Agric Res 2014;9:823–830.

Stevenson JE, Gay K, Barrett TJ, Medalla F, Chiller TM, Angulo FJ. Increase in nalidixic acid resistance among non-Typhi *Salmonella enterica* isolates in the United States from

1996 to 2003. Antimicrob Agents Chemother 2007;51: 195–197.

Van TT, Nguyen HN, Smooker PM, Coloe PJ. The antibiotic resistance characteristics of non-typhoidal *Salmonella enterica* isolated from food-producing animals, retail meat and humans in South East Asia. Int J Food Microbiol 2012;154: 98–106.

Vaz CSL, Streck AF, Michael GB, Marks FS, Rodrigues DP, dos Reis EM, Cardoso MR, Canal CW. Antimicrobial resistance and subtyping of *Salmonella enterica* subspecies enterica serovar Enteritidis isolated from human outbreaks and poultry in southern Brazil. Poult Sci 2010;89:1530–1536.

Voss-Rech D, Vaz CSL, Alves L, Coldebella A, Leão JA, Rodrigues DP, Back A. A temporal study of *Salmonella enterica* serotypes from broiler farms in Brazil. Poult Sci 2015; 94:433–441.

[WHO] World Health Organization. Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR). Critically important antimicrobials for human medicine. 3 ed. 2011. Available at: http://apps.who.int/iris/bitstream/10665/77376/1/9789241504485_eng.pdf Accessed May 26, 2016.

[WHO] World Health Organization. Antimicrobial resistance. Fact sheet No. 194. 2015. Available at: www.who.int/mediacentre/factsheets/fs194/en Accessed May 13, 2016.

Wilson IG. Antimicrobial resistance of *Salmonella* in raw retail chickens imported chicken portions, and human clinical specimens. J Food Prot 2004;67:1220–1225.

Address correspondence to:
Daiane Voss-Rech, BSc
Laboratório de Sanidade e Genética Animal
Embrapa Suínos e Aves
Concordia 89700-991
Brazil

E-mail: daiane.rech@embrapa.br

ANEXO A – COMPROVANTE DA SUBMISSÃO DO MANUSCRITO PARA A REVISTA VETERINARY MICROBIOLOGY

Zimbra **daiane.rech@embrapa.br**

Submission VETMIC_2016_428 received by Veterinary Microbiology

De : Veterinary Microbiology <EviseSupport@elsevier.com> Sex, 30 de Dez de 2016 23:22
Assunto : Submission VETMIC_2016_428 received by Veterinary Microbiology
Para : daiane rech <daiane.rech@embrapa.br>
Responder para : vetmic@elsevier.com

This message was sent automatically. Please do not reply.

Ref: VETMIC_2016_428
Title: Impact of treatments for recycled broiler litter on the viability and infectivity of microorganisms
Journal: Veterinary Microbiology

Dear Mrs. Voss-Rech,

Thank you for submitting your manuscript for consideration for publication in Veterinary Microbiology. Your submission was received in good order.

To track the status of your manuscript, please log into EVISE® at: http://www.evise.com/evise/faces/pages/navigation/NavController.jspx?JRNL_ACR=VETMIC and locate your submission under the header 'My Submissions with Journal' on your 'My Author Tasks' view.

Thank you for submitting your work to this journal.

Kind regards,

Veterinary Microbiology

ANEXO B – AUTORIZAÇÃO DA REVISTA FPD PARA INCLUIR O ARTIGO NA DISSERTAÇÃO

Zimbra **daiane.rech@embrapa.br**

RE: Authorization request FPD-2016-2228

De : Stephen Paul Oliver <soliver@utk.edu> Qui, 29 de Dez de 2016 10:07
Assunto : RE: Authorization request FPD-2016-2228 1 anexo
Para : Daiane Voss Rech <daiane.rech@embrapa.br>

We always grant permission for these situations as long as you acknowledge and give credit to Foodborne Pathogens & Disease where your research was published.

Thanks for considering FPD as an outlet to publish your research.

Happy New Year!

Steve Oliver

From: Daiane Voss Rech [mailto:daiane.rech@embrapa.br]
Sent: Wednesday, December 28, 2016 12:01 PM
To: Oliver, Stephen Paul <soliver@utk.edu>
Subject: Authorization request FPD-2016-2228

Dear Dr. Stephen Oliver,

Recently our article entitled "Antimicrobial Resistance in Non-Typhoidal Salmonella Isolated from Human and Poultry-Related Samples in Brazil: 20-year meta-analysis" was accepted for publication in the FPD. For this opportunity, we are grateful!

This research was carried out during my master's degree course.

Therefore, I would like to request your authorization to include a copy of the manuscript in the final document of the master's degree, which will be available at the Federal University of Santa Maria (library and webpage).

I await your opinion,
Thank you for your attention,

Daiane Voss Rech
Laboratório de Sanidade e Genética Animal
Embrapa Suínos e Aves
Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)
Concórdia - SC