UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

SAFEBED: IMPROVING SAFETY AND SUSTAINABILITY OF RECYCLED MANURE SOLIDS AS BEDDING MATERIAL FOR DAIRY COWS

ANA JOSÉ DE OLIVEIRA NUNES PIRES

Orientador(es): Professora Doutora Maria Manuela Castilho Monteiro de Oliveira

Professor Doutor José Ricardo Dias Bexiga
Professor Doutor David Paulo Fangueiro

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Sanidade Animal

2025

UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

SAFEBED: IMPROVING SAFETY AND SUSTAINABILITY OF RECYCLED MANURE SOLIDS AS BEDDING MATERIAL FOR DAIRY COWS

ANA JOSÉ DE OLIVEIRA NUNES PIRES

Orientador(es): Professora Doutora Maria Manuela Castilho Monteiro de Oliveira

Professor Doutor José Ricardo Dias Bexiga
Professor Doutor David Paulo Fangueiro

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Sanidade Animal

Júri
Presidente: Professor Doutor Luís Filipe Lopes da Costa
Vogais:
- Professor Doutor João Rodrigues Gonçalves Goiana Mesquita
- Professor Doutora Maria Cristina Calhau Queiroga
- Professor Doutor José Luis da Silva Pereira
- Professor Doutora Lélia Maria Marcão Chambel
- Professor Doutora Maria Manuela Castilho Monteiro de Oliveira

2025

DECLARAÇÃO RELATIVA ÀS CONDIÇÕES DE REPRODUÇÃO DA TESE

Nome: Ana José de Oliveira Nunes Pires

Título da Tese ou Dissertação: SAFEBED: IMPROVING SAFETY AND SUSTAINABILITY OF RECYCLED MANURE SOLIDS AS BEDDING MATERIAL FOR DAIRY COWS

Ano de conclusão (indicar o da data da realização das provas públicas): 2025

Designação do curso de Mestrado ou de Doutoramento: Doutoramento em Ciências Veterinárias

Área científica em que melhor se enquadra (assinale uma):

☐ Clínica ☐ Produção Animal e Segurança Alimentar

☐ Morfologia e Função ☒ Sanidade Animal

Declaro sobre compromisso de honra que a tese ou dissertação agora entregue corresponde à que foi aprovada pelo júri constituído pela Faculdade de Medicina Veterinária da ULISBOA.

Declaro que concedo à Faculdade de Medicina Veterinária e aos seus agentes uma licença não-exclusiva para arquivar e tornar acessível, nomeadamente através do seu repositório institucional, nas condições abaixo indicadas, a minha tese ou dissertação, no todo ou em parte, em suporte digital.

Declaro que autorizo a Faculdade de Medicina Veterinária a arquivar mais de uma cópia da tese ou dissertação e a, sem alterar o seu conteúdo, converter o documento entregue, para qualquer formato de ficheiro, meio ou suporte, para efeitos de preservação e acesso.

Retenho todos os direitos de autor relativos à tese ou dissertação, e o direito de a usar em trabalhos futuros (como artigos ou livros).

Concordo que a minha tese ou dissertação seja colocada no repositório da Faculdade de Medicina Veterinária com o seguinte estatuto (assinale um):

1. ☒ Disponibilização imediata do conjunto do trabalho para acesso mundial;
2. ☐ Disponibilização do conjunto do trabalho para acesso exclusivo na Faculdade de Medicina Veterinária durante o período de ☐ 6 meses, ☐ 12 meses, sendo que após o tempo assinalado autorizo o acesso mundial*;

* Indique o motivo do embargo (OBRIGATÓRIO)

Nos exemplares das dissertações de mestrado ou teses de doutoramento entregues para a prestação de provas na Universidade e dos quais é obrigatoriamente enviado um exemplar para depósito na Biblioteca da Faculdade de Medicina Veterinária da Universidade de Lisboa deve constar uma das seguintes declarações (incluir apenas uma das três):

1. É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Faculdade de Medicina Veterinária da Universidade de Lisboa, 15 de julho de 2025

Assinatura: Ana José Pires

"A really strong woman accepts the war she went through and is ennobled by her scars."

Carly Simon

To my grandmother, whose resilience echoes in every one of my achievements, and to my mother, who understands like no one else the challenges of this journey and whose presence was fundamental in helping me complete it. This thesis is a reflection of their collective strength, which has guided me to this moment.

Acknowledgements

O doutoramento que culmina nesta tese foi uma jornada intensa e transformadora, repleta de desafios, aprendizagens e momentos de superação.

No entanto, esta caminhada nunca foi solitária. Foram muitas as pessoas que, de diferentes formas, me ajudaram a chegar até aqui, seja partilhando diretamente esta jornada académica ou caminhando ao meu lado, mesmo sem fazer parte dela. O mérito desta conquista pode ser meu, mas sem todos vós, nunca teria chegado ao fim.

Em primeiro lugar aos meus orientadores, Professora Manuela Oliveira, Professor Ricardo Bexiga e Professor David Fangueiro, agradeço por me acompanharem ao longo deste percurso.À Professora Manuela Oliveira, que acreditou no meu potencial e me deu a oportunidade de realizar este doutoramento, e me permitiu crescer enquanto investigadora, sempre incentivando o desenvolvimento do pensamento crítico e científico. Ao Professor Ricardo Bexiga, pelo conhecimento partilhado e pela exigência que me desafiou a melhorar constantemente. Ao Professor David Fangueiro, pela disponibilidade e contributos técnicos que ajudaram a moldar a este trabalho. Apesar das dificuldades e dos desafios, reconheço o impacto do vosso papel na concretização desta tese e no meu crescimento académico e profissional.

Ao Departamento de Microbiologia e Imunologia , agradeço o apoio e o ambiente proporcionado durante esta jornada. Um especial reconhecimento à Professora Eva Cunha, cujo apoio, como doutorada mais recente, representou para mim uma verdadeira passagem de testemunho. À Dr. Joana Guerreiro, pelos conselhos que me ajudaram ao longo do caminho e pelo contributo essencial no artigo em que é primeira autora, parte integrante desta tese.

Na Faculdade, um agradecimento especial à Patrícia, que me acompanha desde o mestrado e foi uma verdadeira mistura de mana mais velha e mãe de doutoramento. Obrigada pela tua dedicação de longa data, pelo apoio constante e pelos momentos de orientação e carinho que tanto marcaram este percurso. Às minhas manas de doutoramento, Catarina Geraldes e Inês Leites, agradeço a cumplicidade e a presença constante. À Catarina, cuja importância foi sentida mesmo nos momentos em que o apoio era mais discreto, e à Inês, cuja amizade recente trouxe um apoio e uma força inigualáveis.

À minha família, expresso o meu mais profundo reconhecimento. À minha mãe, que entende como ninguém os desafios desta jornada, por já ter trilhado um caminho semelhante, e que esteve sempre presente com conhecimento, amor nos momentos mais difíceis. Ao meu pai, pelo apoio prático e racional, sempre focado em encontrar soluções, mas, acima de tudo, pela maior demonstração de apoio que poderia ter-me dado: a permissão para desistir, caso fosse necessário. Esse gesto, mais do que qualquer outro, deu-me força para continuar. Ao meu

irmão pelo interesse e pela capacidade de ouvir, mesmo sem ter muitos conselhos a oferecer. E à minha tia, pelo interesse demonstrado e pela vontade de ajudar, gestos que nunca deixaram de ser sentidos.

À minha família da Cruz Vermelha, que percorreu este caminho ao meu lado, mesmo sem partilharem desta jornada académica, mas que, com generosidade e dedicação, nunca deixaram de me apoiar. À Dina, que foi uma verdadeira "mãe" ao longo deste percurso, oferecendo conforto e incentivo; ao Neves, cuja vivência prática e apoio incondicional me transmitiram segurança; à Li, que, apesar das dificuldades em lidar com as minhas lágrimas, esteve sempre disponível para me ouvir e ajudar de forma prática; ao Reis, pelo carinho e pelos conselhos, mesmo quando não tinha certezas; ao Pereira, que, mesmo com o pouco tempo disponível, esteve sempre atento e disposto a oferecer ajuda; ao Balegas, que foi um pilar emocional e racional, sempre pronto a encorajar, ouvir e a proteger-me; à Paulinha, pela vontade incansável de encontrar soluções e oferecer conselhos; e aos meus afilhados, Bia e Gonçalo, cujos gestos de apoio trouxeram leveza e força: a Bia, com o seu carinho inabalável, e o Gonçalo, com a sua maturidade e capacidade de ouvir e aconselhar.

Este percurso académico foi também uma jornada de crescimento pessoal. Cada nome aqui mencionado representa uma peça essencial desse crescimento – alguns partilhando diretamente a jornada, outros acompanhando-me ao longo do caminho com apoio e generosidade. Se hoje estou a celebrar esta conquista, é porque nunca fiz esta caminhada sozinha. Obrigada a todos, por sustentarem as minhas forças quando mais precisei e por me permitirem acreditar que, mesmo nos momentos mais difíceis, havia um caminho a seguir. Esta vitória é tanto minha quanto vossa.

Funding

The present work was funded by:

- FCT - PhD fellowship, Portugal, 2021.07175.BD
- CIISA - Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal, Project UIDB/00276/2020
- LEAF—Linking Landscape, Environment, Agriculture and Food— Research Center, Instituto Superior de Agronomia, University of Lisbon, Lisbon, Portugal, Project UIDB/04129/2020 and UIDP/04129/2020
- AL4AnimalS - Associate Laboratory for Animal and Veterinary Sciences AL4AnimalS, LA/P/0059/2020–AL4AnimalS (funded by FCT).

SAFEBED: MELHORAR A SEGURANÇA E SUSTENTABILIDADE DE SÓLIDOS DE ESTRUME RECICLADO COMO MATERIAL DE CAMA PARA VACAS LEITEIRAS

Resumo

A resistência aos antimicrobianos (RAM) e o impacto ambiental da pecuária leiteira representam desafios críticos para a sustentabilidade do setor. A reutilização de sólidos de estrume reciclado (SER) como material de cama oferece benefícios, mas levanta preocupações quanto à segurança microbiana. Este estudo avaliou o impacto da suplementação de biochar no SER para mitigar riscos de RAM, melhorar a segurança microbiana e reduzir emissões ambientais.

Amostras de SER foram recolhidas de uma exploração leiteira e sujeitas a cinco tratamentos: SER não suplementado (C-), acidificado (C+) e suplementado com 2,5%, 5% ou 10% de biochar. As amostras foram incubadas durante 30 dias à temperatura ambiente, com análises aos dias 0, 5, 15 e 30. Foram realizados dois ensaios, um em período húmido (abril-maio) e outro em período seco (junho-julho). A caracterização incluiu quantificação bacteriana, isolamento de *Escherichia coli* e *Enterococcus* spp., perfis de RAM e virulência, sequenciação de 16S rDNA para avaliar alterações na comunidade microbiana e avaliação das propriedades químicas, da retenção de nutrientes e das emissões de gases com efeito de estufa (GEE).

Relativamente à quantificação bacteriana não foram registadas reduções significativas de *E. coli* e *Enterococcus* spp., embora tenha sido observada uma tendência de diminuição na resistência a oxitetraciclina e na formação de biofilme. Em contraste, a analise do microbiona revelou uma redução significativa da carga bacteriana, com 5% de biochar a reduzir 59,50% das contagens sob condições húmidas, embora tenha sido registado um aumento de *Brucella* spp. e *Pseudomonas* spp. Adicionalmente a 10% de biochar reduziu as emissões de CO_2 (32%) e N_2O (47%), estabilizou o azoto e minimizou perdas por volatilização, sem impacto significativo nas emissões de metano. A análise química indicou um aumento de micronutrientes e metais pesados (exceto molibdénio), além de um aumento nos teores de potássio e magnésio e uma redução de sódio, fósforo e carbono orgânico total. Este estudo destaca o potencial do biochar na mitigação da RAM e na sustentabilidade da produção leiteira. No entanto, são necessários estudos adicionais para otimizar as concentrações aplicadas, avaliar efeitos a longo prazo e validar a sua aplicação em larga escala.

Palavras-chave: Biochar, Sólidos de estrume reciclado, Resistência antimicrobiana, Modulação do microbioma, Sustentabilidade ambiental, Emissões de gases com efeito de estufa, Uma-só-saúde

SAFEBED: IMPROVING SAFETY AND SUSTAINABILITY OF RECYCLED MANURE SOLIDS AS BEDDING MATERIAL FOR DAIRY COWS

Abstract

Antimicrobial resistance (AMR) and the environmental impact of livestock production pose significant challenges to sustainable dairy farming. The use of recycled manure solids (RMS) as bedding material offers economic and environmental benefits but raises concerns regarding microbial safety, including the persistence of antimicrobial-resistant bacteria and potential pathogens. This study evaluated the effects of biochar supplementation in RMS to mitigate AMR risks, enhance microbial safety, and reduce environmental emissions.

Fresh RMS samples were collected from a commercial dairy farm and divided into five treatments: non-supplemented RMS (C-), acidified RMS (C+), and RMS supplemented with 2.5%, 5%, or 10% biochar. Samples were incubated for 30 days at ambient temperature, with assessments on Days 0, 5, 15, and 30. Two assays were conducted: one in a humid period (April–May) and another in a dry period (June–July). Microbial analyses included bacterial quantification, *Escherichia coli* and *Enterococcus* spp. isolation, AMR and virulence profiling, and 16S rDNA sequencing for microbial community shifts. In parallel, the environmental study examined RMS characteristics, nutrient retention, and greenhouse gas (GHG) emissions.

Results from the bacterial quantification revealed no statistically significant reduction in *E. coli* and *Enterococcus* spp. counts with biochar supplementation. However, trends suggested a potential decrease in AMR and virulence factors, with reductions observed in oxytetracycline resistance and biofilm formation. In contrast, the microbiome analysis demonstrated a significant reduction in bacterial loads, with 5% biochar leading to a 59.50% decrease under humid conditions. However, *Brucella* spp. and *Pseudomonas* spp. increased in biochar-amended RMS.

Environmentally, the supplementation with 10% biochar significantly reduced CO_2 (32%) and N_2O (47%) emissions, stabilized nitrogen, and minimized volatilization losses. However, methane emissions were largely unaffected. Addicionally, Biochar supplementation increased micronutrient and heavy metal concentrations (except for molybdenum), as well as potassium and magnesium levels, while decreasing sodium, phosphorus, and total organic carbon content.

This study highlights biochar's potential for improving microbial safety and environmental sustainability in dairy farming. Further research should optimize biochar concentrations, assess long-term effects, and evaluate field-scale applications to fully harness its role in sustainable manure management.

Keywords: Biochar, Recycled manure solids, Antimicrobial resistance, Microbiome modulation, Environmental sustainability, Greenhouse gas emissions, One Health

SAFEBED: MELHORAR A SEGURANÇA E SUSTENTABILIDADE DE SÓLIDOS DE ESTRUME RECICLADO COMO MATERIAL DE CAMA PARA VACAS LEITEIRAS

Resumo Alargado

A resistência antimicrobiana (RAM) é reconhecida como uma das principais ameaças globais à saúde pública, que afeta tanto a medicina humana quanto a medicina veterinária. Este problema pode ser agravado pelo uso inadequado de antimicrobianos na pecuária, que exerça uma pressão seletiva sobre os microrganismos presentes no ambiente agrícola, favorecendo o desenvolvimento de bactérias resistentes. No setor leiteiro, a gestão de estrume é um ponto crítico para controlo da disseminação de RAM e da emissão de gases com efeito de estufa (GEE), como metano (CH_4) e óxido nitroso (N_2O), que contribuem significativamente para as mudanças climáticas. Embora algumas explorações promovam o reaproveitamento de sólidos de estrume reciclado (SER) para material de cama para bovinos leiteiros, é necessário que esta prática seja complementada com estratégias inovadoras que contribuam para a melhoraria da sustentabilidade e da segurança microbiológica deste tipo de cama. Entre as estratégias inovadoras, a utilização de biochar, um material rico em carbono produzido por pirólise, tem emergido como uma solução promissora.

Este estudo teve como objetivo avaliar o impacto da suplementação de SER com biochar na segurança microbiológica, na mitigação de GEE e na composição química do SER durante o armazenamento, abordando tanto os desafios microbiológicos quanto os ambientais. O trabalho prático foi estruturado em três componentes principais, com base em ensaios de incubação: análise da dinâmica microbiana focada em bactérias indicadoras, como *Escherichia coli* e *Enterococcus* spp., em termos quantitativos e dos perfis de resistência a antibióticos e de virulência (Capítulo 2); avaliação da diversidade microbiana por metagenómica (Capítulo 3); e monitorização das emissões de GEE e características químicas do SER (Capítulo 4).

Os ensaios foram realizados em dois períodos, húmido (abril-maio) e seco (junho-julho), utilizando amostras de SER de uma exploração leiteira em Portugal, obtidas por separação mecânica. As amostras foram divididas em cinco grupos: Controlo Negativo (C-) - SER não suplementado; Controlo Positivo (C+) - SER acidificado com 10% de ácido sulfúrico; e SER suplementado com 2,5%, 5% ou 10% de biochar (p/p).

Para o trabalho descrito nos capítulos 2 e 3, as amostras foram armazenadas em contentores ventilados durante 30 dias, com recolha de amostras para análise realizada nos dias 0, 5, 15 e 30. Posteriormente, foram realizadas análises de quantificação bacteriana e isolamento de *E. coli* e *Enterococcus* spp., seguidas de testes de resistência a antibióticos e de produção de factores de virulência (Capítulo 2). A análise do microbioma no capítulo 3 foi realizada através da sequenciação do gene que codifica para o 16S rRNA, permitindo a avaliação

detalhada de mudanças na diversidade e composição microbiana entre os tratamentos. A diversidade alfa, que mede a riqueza e uniformidade das espécies dentro de cada amostra, foi avaliada através dos índices de Shannon e uniformidade de Pielou. Já a diversidade beta, que compara a composição microbiana entre diferentes amostras para identificar semelhanças e diferenças na estrutura das comunidades microbianas, foi analisada através da Análise de Coordenadas Principais baseada no índice de Bray-Curtis.

A análise ambiental descrita no capítulo 4 focou-se nas amostras correspondentes ao C-, SER suplementado com 2,5% e SER suplementado com 10% de biochar, sem distinção sazonal, mas mantendo o período do ensaio de 30 dias, utilizando um sistema de monitorização contínua e intermitente de GEE. Além disso, a composição química das amostras foi avaliada no início e fim do período experimental. Para tal, foram monitorizadas as emissões temporais e cumulativas de dióxido de carbono (CO_2), óxido nitroso (N_2O) e metano (CH_4), além de análises de parâmetros químicos como pH, azoto total e matéria orgânica, com o objetivo de avaliar os efeitos do biochar no potencial agronómico do SER.

A suplementação com 5% de biochar resultou numa redução limitada e não significativa nas cargas bacterianas de *E. coli* e *Enterococcus* spp. nas amostras avaliadas na época húmida, enquanto nas amostras avaliadas na época seca não se observaram diferenças consistentes. Para além disso, a avaliação dos perfis de resistência e virulência realizada demonstrou que os isolados de *E. coli* e *Enterococcus* spp. apresentaram resistência a várias classes de antimicrobianos, incluindo tetraciclinas e macrólidos, mas sem diferenças estatisticamente significativas entre os diferentes tratamentos aplicados ao SER. Observou-se que alguns isolados apresentaram capacidade de produção de fatores de virulência, como hemolisina e biofilme, mas também sem alterações significativas associadas à suplementação do SER com biochar. O potencial patogénico dos isolados foi avaliado com base nos índices de resistência antimicrobiana (MAR) e virulência (VIR), e todos os isolados foram classificados como sem risco, exceto os do grupo C+ que foram classificados como de baixo risco. Estes resultados reforçam a necessidade de estudos adicionais para compreender melhor os efeitos da suplementação com biochar relativamente aos perfis de resistência e virulência das espécies bacterianas presentes no SER.

Na análise do microbioma, a aplicação de biochar, particularmente a 10%, demonstrou uma tendência para aumentar a abundância de grupos microbianos benéficos presentes no SER, como Firmicutes e Actinobacteria, enquanto promoveu uma redução na presença de potenciais agentes patogénicos, incluindo membros da Família Enterobacteriaceae. Na época húmida, as amostras de SER suplementadas com 5% de biochar apresentaram uma redução média de 59,50% relativamente a Enterobacteriaceae, *Streptococcus* spp., *Enterococcus* spp. e *Staphylococcus* spp..

Apesar dos resultados promissores mencionados, nem todas as alterações observadas nas amostras de SER suplementadas com biochar foram desejáveis. No ensaio realizado durante a época húmida observou-se um aumento significativo na abundância relativa de dois agentes patogénicos relevantes para bovinos e outras espécies animais, incluindo humanos: *Pseudomonas* spp. e *Brucella* spp. Especificamente, nas amostras de SER suplementadas com 2,5% de biochar, a abundância de *Pseudomonas* spp. aumentou 283,99% e a de *Brucella* spp. 110,25% em relação ao controlo negativo. No ensaio realizado durante a época seca, *Pseudomonas* spp. apresentou uma redução de 31,15% nas amostras de SER suplementadas com 2,5% de biochar, mas os níveis de *Brucella* spp. permaneceram elevados, apresentando um aumento de 67,06% em comparação com o controlo negativo. Estes resultados reforçam a necessidade de maior monitorização para compreender os potenciais riscos associados a esses agentes patogénicos em amostras de SER suplementadas com biochar.

A análise ambiental demonstrou que a suplementação do SER com biochar influenciou significativamente as suas propriedades físico-químicas e as emissões de GEE. O biochar promoveu a estabilização da matéria orgânica, a retenção de nutrientes e a mitigação de emissões de GEE, especialmente quando aplicado em concentrações mais elevadas.

Relativamente às propriedades químicas, a suplementação com 10% de biochar promoveu uma maior estabilização da matéria orgânica e uma redução das perdas de azoto, enquanto 2,5% de biochar reteve mais azoto biodisponível, o que pode ter contribuído para a persistência microbiana observada nos capítulos anteriores. Estes resultados sugerem que a dosagem do biochar desempenha um papel crítico, com concentrações mais baixas a favorecer a retenção de nutrientes e a atividade microbiana, enquanto concentrações mais elevadas podem favorecer a estabilização dos compostos orgânicos, mas sem promover uma supressão microbiana tão eficaz. A análise química revelou ainda que a suplementação com biochar aumentou a concentração de micronutrientes e metais pesados (exceto molibdénio), bem como os teores de potássio (K) e magnésio (Mg) ao fim do período experimental. No entanto, observou-se uma redução nos teores de sódio (Na), fósforo (P) e carbono orgânico total (TOC), sugerindo que o biochar influencia a dinâmica de nutrientes no SER.

A análise ambiental demonstrou também que a suplementação do SER com biochar teve um impacto significativo nas emissões de GEE, os cumulativos das leituras intermitentes mostraram que 10% de biochar reduziu significativamente as emissões de CO_2 em 32% e as de N_2O em 47%, em comparação com o C-. Estes resultados sugerem que o biochar pode mitigar as emissões de GEE associadas ao armazenamento do SER, particularmente no ciclo do azoto. No entanto, as emissões de CH_4 não sofreram alterações significativas. Estes resultados reforçam a complexidade da influência do biochar nas emissões e sugerem que a

sua eficácia na redução dos GEE pode depender de múltiplos fatores ambientais e microbiológicos.

Em geral, os resultados demonstraram tendências promissoras na redução de microrganismos patogénicos, estabilização de nutrientes e mitigação das emissões de GEE, embora nem todas as variáveis tenham sido estatisticamente significativas. Enquanto no Capítulo 2 a suplementação com biochar não levou a reduções significativas de *E. coli* e *Enterococcus* spp. nem mitigou completamente a RAM, no Capítulo 3 a suplementação com 5% de biochar reduziu significativamente algumas bactérias potencialmente patogénicas (59,5%), mas foi acompanhada pelo aumento inesperado de *Brucella* spp. e *Pseudomonas* spp.. O Capítulo 4 revelou melhorias na retenção de azoto e estabilização da matéria orgânica, particularmente com 10% de biochar, além de reduções nas emissões de CO_2 e N_2O, respetivamente.

Este trabalho representa um avanço inicial na avaliação do potencial da suplementação com biochar como solução sustentável para a gestão do SER, mas a integração do biochar em sistemas agrícolas sustentáveis requer a realização de estudos que explorem as concentrações ideais de suplementação, avaliem os impactos a longo prazo da suplementação com biochar na dinâmica microbiana, e investiguem a sua interação com o solo, tanto a nível da microbiota como das características físico-químicas do mesmo. Além disso, será essencial realizar ensaios de campo para capturar as complexidades dos ambientes reais de explorações leiteiras, e validar a eficácia da aplicação do biochar em diferentes cenários. Paralelamente, devem ser desenvolvidos esforços no sentido do estabelecimento de métodos de produção mais económicos, avaliar a viabilidade económica da aplicação do biochar, bem como, o impacto ambiental da sua produção com vista à sua aplicação nos sistemas agrícolas. Uma melhor compreensão destas dinâmicas poderá eventualmente permitir integrar o biochar de forma eficaz em explorações leiteiras e outros sistemas agrícolas, promovendo práticas sustentáveis alinhadas com os objetivos do conceito Uma Só Saúde.

Palavras Chave: Resistência antimicrobiana, Gases com efeito de estufa, Sólidos de estrume reciclado, Biochar, Segurança microbiológica, Diversidade microbiana, Sustentabilidade agrícola, Uma Só Saúde

Table of Contents

List of Figures

List of Tables

List of Abbreviations

2.5B or 2.5%B - Recycled manure solids supplemented with 2.5% Biochar (w/w)
5B or 5%B - Recycled manure solids supplemented with 5% Biochar (w/w)
10B or 10%B - Recycled manure solids supplemented with 10% Biochar (w/w)
AD - Anaerobic digestion
Ag^+ - Silver ions
AgCuNPs - Combined silver and copper nanoparticles
AgNPs - Silver nanoparticles
AHVLA - Animal Health and Veterinary Laboratories Agency
Alg- SDCT - Algorithmic-guided Selective dry cow therapy
AMEG - Antimicrobial Advice Ad Hoc Expert Group
AMPs - Antimicrobial peptides
AMR - Antimicrobial Resistance
AMU - Antimicrobial Usage
ARB - Antimicrobial resistant bacteria
ARG - Antimicrobial resistant genes
AR - First-order autoregressive
Avg - Average
BDCT - Blanket dry cow therapy
BE - Bile Esculin
BFR - Bovine Foot Rot
BHI - Brain Heart Infusion
BLAST - Basic Local Alignment Search Tool
BRD - Bovine Respiratory Disease
C+ - Positive Control
C- - Negative Control
CA-SFM - *Le Comité de l'Antibiogramme de la Société Française de Microbiologie*
CLSI - Clinical & Laboratory Standards Institute
CS - Compound symmetry
CuNP - Copper nanoparticles
Cult-SDCT - Culture-guided Selective dry cow therapy
DM - Dry matter
DS - Dry period
EC - Electrical conductivity
EARS-Vet - European Antimicrobial Resistance Surveillance network in Veterinary Medicine

ECDC - European Centre for Disease Prevention and Control

EFSA - European Food Safety Authority

EHEC - Enterohemorrhagic *E. coli*

EMA - European Medicines Agency

EPI - Environmental Protection Index

ESKAPE - *Enterococcus faecium*, *Staphylococcus. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.

ESVAC - European Surveillance of Veterinary Antimicrobial Consumption

ETEC - Enterotoxigenic strains of *E. coli*

EU - European Union

EUCAST - European Committee on Antimicrobial Susceptibility Testing

FAO - Food and Agriculture Organization

FDA - Food and Drug Administration

FeNPs - Iron nanoparticles

F - Airflow rate

G - Gas concentration

GHGs - Greenhouse Gases

GRAM - Global Report on Antimicrobial Resistance

GWP - Global Warming Potential

IMI - Intramammary infections

IPCC - Intergovernmental Panel on Climate Change

JIACRA - Joint Inter-agency Antimicrobial Consumption and Resistance Analysis

LS - Least squares means

MAC - MacConkey Agar

MAR - Multiple Antibiotic Resistance

MDR - Multidrug Resistant

MIPs - Molecular imprinting polymers

MIPCs - Molecularly imprinted polymer-coated stainless-steel sheets

MR - Milk Replacer

NAS - Non-*aureus* staphylococci

NPs - Nanoparticles

OM - Organic matter

PCA - Principal Component Analysis

PCoA - Principal Coordinates Analysis

PCU - Population correction unit

Persulfate-based-AOPs - Persulfate-based advanced oxidation process methods

PtNPs - Platinum nanoparticles

ROS - Reactive oxygen species

RMS – Recycled manure solids

S/L - Solid-liquid separation

SB - Slanetz and Bartley Agar

SDCT - Selective dry cow therapy

SPRI - Solid Phase Reversible Immobilization

STD - Standard deviation

TOC - Total organic carbon

US - United States of America

VIR - Virulence index

VRE - Vancomycin-resistant enterococci

WHO - World Health Organization

WM - Waste Milk

WOAH - World Organization for Animal Health

WOAH / OIE - World Organization for Animal Health (formerly *Office International des Epizooties*)

WS - Wet period

Chapter I

INTRODUCTION

Ana José Pires, Gonçalo Pereira, David Fangueiro, Ricardo Bexiga, Manuela Oliveira. 2024. When the solution becomes the problem: a review on antimicrobial resistance in dairy cattle. Future Microbiology. 10.2217/fmb-2023-0232

Authors' contributions:

Ana Pires: drafting, critical revision, and final approval of the manuscript.
Gonçalo Pereira: contributed to the revision of the manuscript.
David Fangueiro: contributed to the revision the manuscripts and supervision throughout.
Ricardo Bexiga: helped to draft and revise the manuscripts and supervision throughout.
Manuela Oliveira: critical revision of the manuscripts, final manuscript approval and supervision throughout.

1.General Introduction

1.1 Antimicrobial Resistance: A Global Threat

Antimicrobial resistance (AMR) is a critical global health challenge, affecting human medicine, animal health, and the environment (Baquero, 2021; Lee, 2019). Misuse of antibiotics across human and veterinary medicine accelerates AMR development and dissemination, leading to increased healthcare costs and mortality (Iskandar et al., 2022; Munita & Arias, 2016). The One Health framework highlights the interconnectedness of human, animal, and environmental health, so AMR is classified as a major threat for the safeguarding of One Health, as it can spread between these domains through direct and indirect contact, and also through food products and environmental contamination (de Greeff et al., 2022; Oliver et al., 2011; Richardson et al., 2018)

1.2 The Role of Dairy Farming in AMR and Environmental Impact

Dairy farming may significantly contribute to AMR development and dissemination and environmental degradation. Antibiotics used to treat livestock can exert selective pressure promoting antimicrobial resistant bacteria (ARB) in manure, which may then spread to humans through direct contact, food consumption, or environmental contamination (de Greeff et al., 2022; Oliver et al., 2020). Manure decomposition also emits greenhouse gases (GHGs) like methane and nitrous oxide, impacting climate and biodiversity (Fangueiro et al., 2018; Silva & Cabrera, 2024; US-EPA, 2006).

Various manure treatments, like anaerobic digestion, composting, and acidification, contribute for a reduction in emissions, but also present some limitations, such as infrastructure costs and not acting on the reduction of resistant bacteria (Fangueiro et al., 2015; Marutescu et al., 2022; Yan et al., 2024)

Biochar, a carbon-rich material produced via pyrolysis, offers a promising alternative for addressing AMR, bacterial load, and environmental impacts in manure management (Meyer et al., 2011; Zhang et al., 2014; Y. Zhang et al., 2023). With its high surface area, porosity, and adsorption capacity, biochar can capture pathogens and antibiotic residues, thus reducing bacterial load and minimizing AMR spread (Ma et al., 2024) Additionally, biochar mitigates GHG emissions by adsorbing ammonia, methane (CH_4), and nitrous oxide, contributing to lower carbon footprints in dairy farming (Ma et al., 2024; Sapkota et al., 2024). Its nutrient-stabilizing properties further enhance the agronomic value of treated manure, aligning with sustainable agriculture goals and climate resilience (Bello et al., 2020; Du et al., 2023; Sapkota et al., 2024).

1.3 Biochar-supplemented Recycled Manure Solids for improving safety and sustainability of bedding material for dairy cows

This research aimed to evaluate the potential of biochar supplementation of Recycled Manure Solids (RMS) to ensure microbial and environmental safety and environmental for RMS use in dairy farming. The biochar influence on the microbiome and microbiota present in RMS was tested, focusing on *E. coli* and *Enterococcus* species, by characterizing their antibiotic resistance and virulence profiles. Additionally, the study assessed biochar's environmental impacts, analyzing greenhouse gas emissions, chemical properties, and nutrient composition. By approaching biochar supplementation from microbial, environmental, and nutrient perspectives, this research aimed to contribute to sustainable and safe manure management practices within dairy farming.

This first chapter opens with an introduction to the issue of AMR in dairy farming, examining its transmission pathways and environmental impact, particularly associated with manure management. This is followed by a literature review that explores AMR mechanisms, the limitations of existing manure treatments, and the potential role of biochar as a sustainable solution to reduce bacterial load, mitigate AMR risks, and address environmental challenges in dairy farming.

Chapters 2 and 3 were based on an incubation experiment conducted with fresh RMS samples collected from a dairy farm in Elvas, Portugal. RMS samples were divided into five treatment groups: a non-supplemented negative control, RMS supplemented with 10% H_2SO_4 as a positive control, and three RMS groups supplemented with biochar at 2.5%, 5%, and 10% concentrations. After storage in naturally-ventilated containers over a 30-day period during two seasonal phases (humid and dry seasons), samples from each group were collected at four time points for further characterization. This experimental setup provided the foundation for analyzing microbial dynamics, resistance patterns, and the effects of biochar supplementation in RMS.

In light of biochar's potential benefits and the cost-effectiveness of using RMS as cow bedding, in Chapter 2, we characterized biochar-supplemented RMS by evaluating its potential to limit the spread of pathogenic and antimicrobial-resistant bacteria before its broader use as cow bedding. Isolates of *Escherichia coli* and enterococci were identified from all treatment conditions of the incubation experiment and subsequently analyzed for their antimicrobial susceptibility and virulence profiles to determine any biochar-related effects across all conditions. investigated biochar's effects on the microbial community within RMS. Isolates of *E. coli* and enterococci were identified from all treatment conditions of the incubation experiment and then characterized regarding their antimicrobial susceptibility and virulence profiles to determine any biochar-related effects across all conditions. Monitoring *E. coli* and enterococci is essential, as they serve as reliable indicators of fecal contamination and can

signal potential health hazards within bedding material. *E. coli* is not only linked to infections like mastitis in cattle but also poses zoonotic risks with strains such as Enterohemorrhagic *E. coli* (EHEC), known to cause severe gastrointestinal illnesses in humans (Kawasaki & Ambrosini, 2024). Enterococci, widely distributed in the environment, play a role in both animal and human infections and are central to AMR monitoring due to their involvement in nosocomial infections (Barlow et al., 2017; Ekore et al., 2022). Moreover, European regulations require that manure by-products, including RMS, maintain an Enterobacteriaceae threshold of no more than 1000 CFU/g to ensure microbial safety (Galama et al., 2020; Heinonen-Tanski et al., 2006).

The comprehensive evaluation of AMR and virulence profiles in indicator bacteria isolated from biochar-supplemented RMS provides essential insights into the safety and feasibility of biochar as an amendment for the bedding material, ultimately supporting sustainable practices that reduce the risk of AMR transmission and protect both animal and public health.

Building on the microbial and AMR findings of Chapter 2, Chapter 3 investigated biochar's effects on the broader microbial community within RMS. Using high-throughput 16S rDNA sequencing, the research described in this chapter focused on assessing shifts in bacterial populations present in each RMS group under evaluation, particularly targeting potential cow pathogens such as Enterobacteriaceae, *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Salmonella*, and *Brucella* species. This information is essential, as RMS can host significant bacterial populations, including potential pathogens and antimicrobial-resistant bacteria (ARB), representing risks for both environmental contamination and animal health. It was observed that cattle manure hosts a dense and diverse microbial population, commonly including *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, with high levels of Enterobacteriaceae, *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridioides difficile*, which often harbor antimicrobial resistance genes (Buta-Hubeny et al., 2022; Heinonen-Tanski et al., 2006; Sukhum et al., 2021). Moreover, some of these bacteria are commonly associated with mastitis. So, although RMS has become a popular and cost-effective bedding material for use in dairy farms, this use also raises health concerns. Bedding materials play a critical role in dairy cow health, as prolonged exposure to pathogens within bedding can lead to infections, particularly mastitis, which remains one of the most significant sources of economic loss in dairy operations (Klaas & Zadoks, 2018; Rowbotham & Ruegg, 2016).

Finally, Chapter 4 investigated the environmental impact of biochar on RMS. A small-scale experiment was conducted under controlled conditions, with RMS collected from a dairy farm near Lisbon and separated into the same five treatment groups as in chapter 2 and 3: Each treatment was stored in sealed containers for 30 days under controlled conditions, with continuous monitoring of methane, nitrous oxide, and carbon dioxide emissions. Moreover,

the chemical properties, including pH, nitrogen forms, carbon content, and nutrient composition, were measured at the start and end of the experiment.

Reducing GHG emissions is a priority within the European Union's climate strategy, to which the manure management at the dairy sector is a significant contributor, particularly for methane and nitrous oxide emissions (The European Green Deal, 2019). It is critical to address these emissions, as they contribute both to climate change and to local air quality issues, thus heightening the need for improved practices in manure management (Fangueiro et al., 2021).

Amendment with biochar has emerged as a promising option for environmental remediation, with the potential to reduce harmful emissions and enhance the agronomic value of RMS (Akdeniz, 2019; Bello et al., 2020; Du et al., 2023; Ma et al., 2024).

Overall, this project comprehensively evaluated the potential of biochar-supplemented RMS to be used as a sustainable bedding material in dairy farming, assessing biochar effects on microbial safety, AMR transmission risks, and environmental impact.

2. AMR in Dairy Farms

2.1 When the solution becomes the problem: a review on antimicrobial resistance in dairy cattle.

Abstract:

Antibiotics' action, once a "magic bullet," is now hindered by widespread microbial resistance, creating a global antimicrobial resistance (AMR) crisis. A primary driver of AMR is the selective pressure from antimicrobial use.

Between 2000 and 2015, antibiotic consumption increased by 65%, reaching 34.8 billion tons, 73% of which used in animals. In the dairy cattle sector, antibiotics are crucial for treating diseases like mastitis, posing risks to humans, animals, and potentially leading to environmental contamination.

To address AMR, strategies like selective dry cow therapy, alternative treatments (nanoparticles, phages), and waste management innovations are emerging. However, most solutions are in development, emphasizing the urgent need for further research to tackle AMR in dairy farms.

Plain language summary:

Antibiotics were once seen as a miracle cure for bacterial infections, but now they are losing their effectiveness due to antimicrobial resistance (AMR). This phenomenon is mainly caused by the abuse and misuse of antibiotics in both human and veterinary medicine, especially in animal production.

In the dairy cow industry, the use of certain antibiotics to treat diseases like mastitis is a big concern. To tackle this problem, different approaches are being pursued, including the establishment of practices aiming at the responsible use of antibiotics, the development of alternative treatments such as nanoparticles or specific viruses, and the discovery of better methods to deal with animal waste. However, most of these ideas are still being studied, rendering research to stop AMR from spreading, not only in dairy farming but also globally, urgent, and mandatory.

In conclusion, AMR is a big problem which resolution requires the contribution of all societal settings, including human doctors, veterinarians, researchers, and the general population. Only this way all One Health settings can be protected from this "Silent Pandemic".

Keywords: Dairy Cattle, Antimicrobial Resistance, Resistance Dissemination, Alternative antimicrobial strategies, One-Health

2.1.1 Introduction

Before the beginning of the 20th century, several infectious diseases were responsible for high mortality rates, including pneumonia, typhoid fever, tuberculosis, typhus, syphilis, and the plague, with humans presenting a life expectancy of around 47 years (Baquero, 2021; Lee, 2019; Riley, 2005). Fortunately, the world has evolved since then, with the increase in average life expectancy being mainly associated with the development of better hygiene practices and major scientific breakthroughs. The discovery of antibiotics was an important step towards the control of bacterial infections (Iskandar et al., 2022; Lee, 2019), however, in response to their use, microorganisms developed several resistance strategies, based on a panoply of mechanisms (Munita & Arias, 2016). Shortly after penicillin's discovery in the 1940s, a penicillinase-producing *Escherichia coli* strain with the ability to inactivate penicillin was reported, and, only two years later, penicillin-resistant *Staphylococcus aureus* strains were isolated from hospitalized patients. The uprising of resistance continued throughout the years, in tandem with antibiotics use, with colistin resistance mediated by plasmids being reported in 2000, and resistance to ceftriaxone, a third-generation cephalosporin being reported in 2010 (Sharma et al., 2022) . Furthermore, resistance to one of the most recent antibiotic combinations, Ceftazidime-avibactam, was reported in 2015, only one year after its commercialization (Browne et al., 2020).

Antimicrobial resistance (AMR) can be defined as intrinsic, when bacteria traits, such as the cell wall of Gram-negative bacteria, render them resistant to antimicrobials. Alternatively, this resistance can derive from genetic mutations or the acquisition of new genetic material through horizontal transfer (transduction, conjugation, and transformation), which is defined as acquired resistance (Bairán et al., 2020; Gabibov et al., 2020; Iskandar et al., 2022; Munita & Arias, 2016). As such, several antimicrobial resistance genes (ARG) can be present in the bacterial genome (reflecting genotypic resistance). When an antibiotic exerts its selective pressure on these genes, it can promote the activation of several mechanisms, including efflux pumps expression, cell wall recycling, porin reduction, target protein alteration, and biofilm formation, which participate in bacterial resistance to several compounds (Munita & Arias, 2016). In some cases, resistance ability may be reversible if antibiotic pressure disappears. This occurs due to alterations in membrane permeability, or in the activity of regulators involved in bacterial response to antimicrobials and related stressors. It has been shown that, after being exposed to an environment without antibiotics, drug resistance presented by some bacterial species can be reduced after 480 generations, albeit the rate of loss of resistance may vary (Kernéis et al., 2020).

Antibiotic-resistant bacteria pose a major global hazard to human and animal health. In the 2022 GRAM's Global Burden Report, infections by resistant bacteria are estimated as being responsible for 4,95 million deaths that occurred in 2019 (Vos et al., 2020), surpassed only by

cardiovascular diseases, responsible for 18,56 million deaths, and cancer, responsible for 10,08 million deaths. Of particular concern are the infections promoted by a group of pathogens classified by the World Health Organization (WHO) as of critical, high, and medium priority regarding antimicrobial resistance (Lobanovska & Pilla, 2017). *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (ESKAPE) are included in this group, due to their high level of resistance to most antibiotics and ability to disseminate resistance determinants between all One Health compartments (Bairán et al., 2020). As such, these pathogens pose a serious threat to both human and animal health, prompting the need for the development of new antimicrobial strategies.

Despite worldwide recommendations regarding the risk of antibiotic use and misuse, between 2000 and 2015 the global antimicrobial usage (AMU) rose 65 %, from 21.1 billion to 34.8 billion tons. According to estimates, by 2030 the global consumption of antibiotics will increase by up to 200%, in comparison with the 42 billion daily doses administered in 2015 (Klein et al., 2018; Zhu et al., 2021). Of these, 73% are applied to animals to prevent and treat infections, but also to improve weight gain and productivity (Tiseo et al., 2020; Van Boeckel et al., 2017). Despite antimicrobials use as growth promoters being forbidden in Europe (Brown et al., 2017), a study by Tiseo *et al*. predicted an increase in the global consumption of antimicrobials used for animal health of 11.5% by 2030, from 93,309 tones in 2017 to 104,079 tones in 2030 (Tiseo et al., 2020). Considering the safeguarding of animal health and the livelihood of billions of people who depend on animals for subsistence, the rise of antimicrobial usage and resistance in animals is a significant concern (Criscuolo et al., 2021), especially since human infections involving drug-resistant bacteria can be prompted by animal-to-human transmission (Tabaran et al., 2022; Tang et al., 2017; Titouche et al., 2022; Woolhouse et al., 2015; Xin et al., 2022).

Dairy farming is mainly responsible for milk production, but male calves born on dairy farms and adult cows at the end of their productive life are also used for meat production. Due to the rapid growth of human populations, and consequently increase in the demand for safe food products of animal-origin, an increase in the use of antimicrobials in production animals can also be expected. This may lead to some concerns regarding the role of dairy farms in antimicrobial resistance dissemination (Sharma et al., 2017).

This review employed a comprehensive methodology to gather and synthesize information on the intricate relationship between antibiotic usage, AMR, and the challenges faced by dairy farming. The literature consulted for this review was primarily obtained after an extensive search conducted on Scopus to identify relevant articles, research papers, and reviews. Additionally, legitimate scientific sources, such as reports from reputable organizations, were consulted to ensure the inclusion of diverse perspectives and up-to-date information on the

subject. The search criteria were broad, encompassing expressions such as antibiotic consumption trends, global patterns of antimicrobial resistance, the impact of antibiotic use in cattle, resistance genes in cattle diseases, and strategies to combat AMR in dairy farming. The main keywords used, alone or in combination, were AMR, dairy farms, resistance genes, alternative treatments, manure, and milk. This search originated 532 articles which were narrowed down to the 167 referenced here.

This review aimed to explore the burden of AMR and antibiotic usage in animal production, particularly in dairy farms, as well as characterize the potential impact of the use of antibiotics on dairy farms on AMR development and point out the strategies in place or forthcoming to tackle this issue.

2.1.2 The influence of antibiotic Usage in farm animals on human health – a One-health Perspective.

The potential connection between AMU and AMR is a noteworthy concern for both the scientific community and governmental authorities, particularly when evaluated through a One-health perspective. Furthermore, when trying to determine the exact link between antimicrobials usage and the development of resistance by different bacterial species, the number of factors to be studied and the limitations involved, make this issue even more complex. This complexity makes it difficult to consistently establish a clear connection between AMU and AMR.

For instance, Richardson *et al.* (2018) conducted a study involving *S. aureus* isolates from samples collected in 50 different countries, aiming to explore the dissemination of AMR among various hosts (Richardson et al., 2018). The ability of this bacterial pathogen to circulate between hosts is not new and can be traced back to the Neolithic period when animals were first domesticated. However, the intensification of livestock farming practices has created more opportunities for pathogens to spread between different hosts. According to Richardson *et al*., cows may act as the primary animal reservoir for the emergence of *S. aureus* epidemic clones responsible for human infections. According to these authors, intensive farming directly influences the emergence of AMR in response to the selective pressure associated with the use of antibiotics (Richardson et al., 2018).

Conversely, a collaborative report from multiple agencies, Joint Inter-agency Antimicrobial Consumption and Resistance Analysis (JIACRA) III (European Food Safety Authority et al., 2021), involving the European Centre for Disease Prevention and Control (ECDC), the European Food Safety Authority (EFSA), and the European Medicines Agency (EMA), did not yield conclusive findings regarding the relationship between AMU and AMR, nor regarding the transmission of resistant bacteria between humans and animals. For instance, this report states that an association between AMU and AMR can be observed in *E. coli* from both

humans and animals regarding nearly all antimicrobial classes; on another end, for *Campylobacter jejuni*, the same association was only observed in the animal sector, but not in human settings; finally, regarding *Salmonella*, such association was not evident in neither in humans nor animals. In this report, differences between results were attributed to the typically larger datasets available for *E. coli* and *C. jejuni* when compared to *Salmonella*, and to various limiting factors, such as the inconsistency and variability of available data. The authors of the report concluded that further studies are required for a comprehensive understanding of the association between AMU and AMR, and emphasized the importance of taking additional measures to reduce AMU, predicting that such measures could have a positive impact on the emergence of AMR (European Food Safety Authority et al., 2021).

While a consistent direct link between AMU, AMR, and interspecies resistance transmission is not consistently reported, the majority of studies suggest that a comprehensive global strategy offers the greatest potential to effectively tackle AMR. The collective efforts to control and reduce the use of antimicrobials in both human and veterinary medicine could potentially mitigate overall AMR resistance, emphasizing the importance of the One-Health approach (de Greeff et al., 2022; European Food Safety Authority et al., 2021; Richardson et al., 2018).

2.1.3 Antibiotic Usage in Cattle

Worldwide data on AMU in dairy cattle is sparse and difficult to compile due to the lack of updated databases and the limited number of studies available. The latest study by Tiseo *et al.,* (2020) reports data from 2017 regarding 41 countries, stating that the total amount of antimicrobials administered to food-producing animals worldwide in that year was 93,309 tons. According to that study, which specifies antibiotic usage in different species, cattle was the animal production setting in which the lowest quantity of antimicrobials was used (42 mg/population correction unit - PCU), followed by poultry (68 mg/PCU), and swine production (193 mg/PCU) (Tiseo et al., 2020).

A study by Van Boeckel *et al*. (2017) showed that there is a high variation between the number of antimicrobials used in different countries, ranging from 8 mg/PCU in Norway to 318 mg/PCU in China. As such, the countries with the higher consumption of antimicrobials in both relative (per PCU) and absolute terms should have a significant role in addressing AMR (Van Boeckel et al., 2017). Nevertheless, several countries and regions, namely the European Union (EU) and the United States (US), already monitor the sales of antimicrobials to be used in animals, especially those graded as of critical relevance for human health (European Medicines Agency., 2022).

According to sales statistics reported by the US Food and Drug Administration (FDA), approximately 41% of the antimicrobials sold for administration to cattle correspond to medically relevant antimicrobials, as well as 42% of those used in pigs, 11% in turkeys, and

3% in chickens, (Food and Drug Administration, US, 2022). Nevertheless, from 2016 to 2021, the sales of antibiotics for use in cattle decreased by 32% in the US, as seen in Table 1. Table 2 displays the antimicrobial sales by class during this time period.

Table 1 - Total sales of antimicrobials for cattle administration in the USA in 2021 - The data presented in this table is adapted from the Summary Report on Antimicrobials Sold or Distributed for use in Food-producing Animals, FDA, 2021 (Food and Drug Administration, US, 2022).

	2021 Total Sales (kg) in 2021	Percentage of Total sales	% Change between 2016 and 2021
Medically Relevant	2 460 766	41%	-32%
Non-Medically Relevant	3 290 231	64%	4%

Table 2 - Antimicrobial drugs sold in the USA from 2016-2021 for cattle use: domestic sales and distribution data reported by drug class estimated sales - The data presented in this table is adapted from the Summary Report on Antimicrobials Sold or Distributed for use in Food-producing Animals, FDA, 2021 (Food and Drug Administration, US, 2022).

Antimicrobial Class	2016 Estimated Annual Totals (kg)	2017 Estimated Annual Totals (kg)	2018 Estimated Annual Totals (kg)	2019 Estimated Annual Totals (kg)	2020 Estimated Annual Totals (kg)	2021 Estimated Annual Totals (kg)	% Change 2016 - 2021	% Change 2020 - 2021
Aminoglycosides	161,646	124,675	133,842	139,445	174,132	177,173	10%	2%
Amphenicols	*	*	*	*	47,609	50,732	**	**
Cephalosporins	24,677	23,512	25,337	24,158	21,007	21,197	-14%	1%
Fluoroquinolones	*	*	*	12,560	12,446	12,086	**	**
Lincosamides	118,916	128,642	104,527	114,398	128,562	158,036	33%	23%
Macrolides	194,811	274,479	274,837	286,438	247,581	303,371	56%	23%
Penicillins	99,935	96,936	96,591	78,887	82,008	66,347	-34%	-19%
Sulfonamides	234,955	196,902	187,603	197,486	161,220	136,147	-42%	-16%
Tetracyclines	2,840,519	1,560,542	1,732,416	1,741,883	1,703,391	1,693,680	-40%	-1%
* Not enough data available - less than three sources **Not enough data available - less than three sources – unable to determine the corresponding percentage								

In the EU, the latest report by the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) (European Medicines Agency., 2022) presents data on the sales of antimicrobials up to 2021. The total sales in all 31 countries that submitted data for the report corresponded to 84.4 mg/PCU. Between 2020, in which the total sales corresponded to(88.8 mg/PCU, and 2021 a decrease of 4.9 % in sales was observed. However, a significant disparity between sales in different countries was reported, ranging from 2.5 mg/PCU to 296.5 mg/PCU. The most predominant antibiotic class sold was penicillins, accounting for 31.2% of total sales (26.3 mg/PCU). This class, along with tetracyclines (21.8 mg/PCU, 25.8%), and sulphonamides (8.3 mg/PCU, 9.9%), accounted for 66.9% of all antimicrobial sales in 2021. In general, the sales of compounds from different classes varied greatly between countries. This was also true for the antibiotics from category B, established by the Antimicrobial Advice Ad Hoc Expert Group (AMEG). Sales for compounds from this group ranged from 0.01 to 0.5 mg/PCU for 3rd-generation cephalosporins, 0.01 to 14.8 mg/PCU for 4th-generation cephalosporins, 0.01 to 0.69 mg/PCU for fluoroquinolones and other quinolones, and 0.01 to 12.7 mg/PCU, for polymyxins (European Medicines Agency., 2022). As seen in Table 3, which reports antimicrobials sold in 2021 for administration to cattle, France, and Germany appear as the main consumers of these compounds.

Table 3 - Estimated PCU (in 1,000 tones) of antimicrobials sold in 2021 for administration to cattle per EU country. This table was adapted from the report of Sales of veterinary antimicrobial agents in 31 European countries in 2021 by the European Medicines Agency (European Medicines Agency., 2022).

Country	**Cattle**	**Total**
Austria	420	945
Belgium	473	1,770
Bulgaria	111	391
Croatia	96	331
Cyprus	29	152
Czechia	290	709
Denmark	378	2,452
Estonia	58	114
Finland	203	492
France	2,961	6,758
Germany	2,838	8,071
Greece	74	1,100
Hungary	191	846
Iceland	19	145
Ireland	1,298	2,196
Italy	1,469	3,813
Latvia	85	153
Lithuania	154	297
Luxembourg	42	54
Malta	4	15
Netherlands	1,079	3,092
Norway	215	2,197
Poland	1,538	4,417
Portugal	212	1,063
Romania	733	2,943
Slovakia	83	230
Slovenia	100	184
Spain	1,009	8,245
Sweden	284	788
Switzerland	460	810
United Kingdom	1,716	7,054
Total 31 countries	**18 623**	**61 825**
Percentage	**330%**	**1100/%**

Several independent studies were also performed in developing countries aiming to quantify and understand antibiotic usage in food production in these regions (Chauhan et al., 2018; Dankar et al., 2022; Mshana et al., 2021; Olasoju et al., 2021; Sharma et al., 2022); however, the information available from farms and statistical data provided is insufficient to allow a comprehensive view of the reality of antimicrobials use in these countries. However, a substantial increase in antimicrobial use in livestock is anticipated, particularly in emerging nations such as Nigeria and Indonesia, where an increase in its use exceeding 200% is projected (Dankar et al., 2022). The overuse of antibiotics in developing countries can be associated with the increasing pressure to meet the population's demands for safe food in conjunction with the lack of access to veterinarians, easy accessibility to antibiotics, and a low knowledge of the risks associated with AMR (Chauhan et al., 2018; Dankar et al., 2022; Mshana et al., 2021; Olasoju et al., 2021).

2.1.4 Dairy cattle diseases and AMR

When reviewing antimicrobial use in dairy farms, it is of paramount importance to take into consideration not only milk-producing animals and their related illnesses (e.g., mastitis and metritis) but also those that affect young stock. Approximately 11.73% of the total number of antimicrobials used on dairy farms are administrated to calves, to treat frequent diseases such as bovine respiratory disease and diarrhea (Abdallah et al., 2022; Zhang et al., 2022).

2.1.4.1 Mastitis

Mastitis, defined as the inflammation of the mammary gland generally resulting from microbial infection, is the most common disease affecting dairy cattle, being responsible for a major financial burden worldwide (Ajose et al., 2022; Doehring & Sundrum, 2019, 2019; Down et al., 2017; NAHMS, 2018; Nakada et al., 2023; Naranjo-Lucena & Slowey, 2023). The microorganisms most commonly involved in bovine mastitis are *S. aureus,* as well as other non-*aureus* staphylococci (NAS), *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *E. coli* (Naranjo-Lucena & Slowey, 2023).

2.1.4.1.1 Staphylococcus

Regarding staphylococci, *S. aureus* is a common commensal microorganism with the ability to become an opportunistic pathogen, leading to superficial and invasive infections both in humans and animals, and being able to survive within the mammary gland. On the other hand, NAS are opportunistic invaders, with infections promoted by this group of staphylococci being generally associated with environmental contaminations (Naranjo-Lucena & Slowey, 2023).
Pathogens belonging to this genus express a variety of virulence factors, namely surface proteins responsible for bacteria's capacity to adhere to surfaces, form biofilms, and invade

epithelial or immune cells (Naranjo-Lucena & Slowey, 2023).

S. aureus is intrinsically resistant to ciprofloxacin, daptomycin, gentamicin, linezolid, oxacillin, or vancomycin. It has been documented that this bacterial species exhibits resistance and diminished susceptibility to quinolones and lincomycin, respectively, linked to the activation of efflux pumps. Additionally, NAS are intrinsically resistant to novobiocin (Naranjo-Lucena & Slowey, 2023).

2.1.4.1.2 Streptococcus

Streptococcus is a bacterial genus composed of Gram-positive and catalase-negative cocci, responsible for intramammary infections (IMI) resulting in both clinical and subclinical mastitis (Wyder et al., 2011).

S. uberis became the most frequently reported mastitis pathogen, being mainly associated with the farm environment, with housed cows being at greater risk of developing an *S. uberis* infection than those at pasture. The same is observed for other environmental streptococci, such as *S. dysgalactiae*. Together, *S. uberis* and *S. dysgalactiae* are the most frequently reported cause of mastitis in Ireland, France, Sweden, and Finland. Formerly a prevalent pathogen, *S. agalactiae,* an obligate udder pathogen*,* has been less frequently described due to the continuing improvement of milking management practices, being more common in Portugal and Germany (Naranjo-Lucena & Slowey, 2023; Wyder et al., 2011).

Typically, streptococci have low-level intrinsic resistance to quinolones due to the overexpression of ABC efflux pumps, and to aminoglycosides, due to being facultative anaerobes. These pathogens also present intrinsic resistance to fusidic acid (Naranjo-Lucena & Slowey, 2023).

2.1.4.1.3 Enterobacteriaceae: *E. coli* and *Klebsiella pneumoniae*

The Enterobacteriaceae family includes beneficial commensal microbiota, opportunistic pathogens, and major pathogens. This family is composed by Gram-negative, non-spore-forming, facultative anaerobes that ferment glucose and other sugars and reduce nitrate to nitrite, being catalase positive and oxidase negative (except for *Plesiomonas*). Members of this family are often referred to as enteric bacteria since the main habitat of most of them is the lower gastrointestinal tract of humans and animals (Donnenberg, 2015). From those, *E. coli* and *K. pneumoniae* are the species most often associated with mastitis and, therefore, the ones more explored in this review (Naranjo-Lucena & Slowey, 2023).

E. coli is a well-known opportunistic pathogen that can cause IMI in cattle, with a high clinical impact in animals with immunosuppression. Despite *K. pneumoniae* being a less common mastitis pathogen, it can cause severe clinical mastitis, inducing massive inflammation and necrosis of the mammary gland, and potentially death. Moreover, *E. coli* and *K. pneumoniae*

are crucial in terms of public health and surveillance, as they can serve as a reservoir for antibiotic resistance determinants (Naranjo-Lucena & Slowey, 2023).

Due to the inability of macrolides, aminocoumarins, or glycopeptides to penetrate the outer membrane of Gram-negative bacteria, *Enterobacteriaceae* are considered intrinsically resistant to these antimicrobial classes (Klobucar & Brown, 2022). Still, some exceptions have been reported, including the macrolide azithromycin, which has been found to be effective against *E. coli* (Gomes et al., 2019).

2.1.4.2 Reproductive diseases

Due to the trauma of the birth canal, placental detachment, systemic inflammation, metabolic stress, immune suppression, and shifts in the uterine microbiota that occur in the early postpartum period, dairy cows are susceptible of developing reproductive tract inflammatory diseases (Bogado Pascottini et al., 2023).

Metritis, particularly puerperal metritis, is the second most common reason for antimicrobial treatment of dairy cattle (Garzon et al., 2022). This inflammatory disease has a complex etiology, with several bacteria having already been associated with post-partum infections, such as *E. coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum,* and *Prevotella melaninogenica. T. pyogenes* is also responsible for severe cases of metritis, as well as for mastitis, therefore very relevant in terms of postpartum dairy cows' medicine. Considered one of the most frequent causes of antibiotic resistant mastitis and metritis, this bacterial species will be our focus in this review (Rezanejad et al., 2019).

2.1.4.2.1 Trueperella pyogenes

T. pyogenes is a Gram-positive, non-motile, β-hemolytic, and widespread opportunistic pathogen, frequently present in the mucus layer of upper respiratory, urogenital, and gastrointestinal tracts of livestock. Additionally, this species can act as a primary pathogen following different traumas, being a causative agent of metritis, abortion, mastitis, infertility, and pneumonia in dairy herds (Rezanejad et al., 2019). The resistance determinants previously reported in this bacterial species are summarized in Table 4.

Table 4 - Summary of resistance genes profiles reported for bacteria associated with diseases in dairy cattle. Resistance to β-lactamic antibiotics: β-lactamase *bla* genes. Resistance to methicillin and oxacillin: *mec* genes; Resistance to Tetracyclines: *tet* genes; Resistance to Macrolides or Lincosamides or Streptogramins: *erm*, *ere*, *lnu*, *lsa*, *MLS*$_b$, *msr*, *mph*, *vga*, and *sal* genes; Resistance to Aminoglycosides and Aminocyclitols: *aph*, *aad*, *ant*, *str* and *dfr*A1*aad*A1 genes; Resistance to trimethoprim: *dfr* genes; Resistance to Quinolones: *gyr*, *par*, *qnr* and *flo*R genes; Resistance to chloramphenicol: *cat* genes; Resistance to sulfonamides: *sul* genes, Resistance to colistin: *mgr* gene; Resistance to aminoglycoside: *orf* and *str*.

Disease	**Bacterial species**	**Resistance gene**	**Ref.**
Mastitis	*Staphylococcus* spp.	*bla*$_{ARL}$, *bla*$_Z$, *mec*A, *mec*C, *tet*(K), *tet*(L), *tet*(38), *tet*(M), *aph*A3, *aad*E, *ant*(6)-Ia, *str, dfr*A1*aad*A1, *aad*A2, *dfr*A12-*orf*X2-*aad*A2, *aad*A, *erm*(A), *erm*(B), *erm*(C), *erm*(T), *msr*(A), *msr*(B), *ere*(A), *mph*(C), *lnu*(A), *lnu*(B) *vga*(A), *vga*(C), *lsa*(E), *sal*(A), *dfr*(A), *dfr*(D), *dfr*(G), *dfr*(K) *par*E and *gyr*B	(Naranjo-Lucena & Slowey, 2023)
	Streptococcus spp.	*bl2b, bla*Z, *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), *aph*A-3, *aad*-6 *erm*(A), *erm*(B), *mph*(B), *lnu*A, *lnu*B, *lnu*(D), *lnu*(C), *cat*1, *cat*2, *sul*1, *sul*2 and *sul*3	
	E. coli	*bla*$_{TEM}$-, *bla*$_{SHV-1}$, *bla*$_{SHV-2}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-55}$, *bla*$_{CTX-M-96}$, *bla*$_{SHV-12}$, *bla*$_{CMY-59}$, *bla*$_{NDM-1}$, *bla*$_{NDM-5}$, *tet*(A), *tet*(B), *tet*(C), *tet*(E), *tet*(G), *rmt*B, *aac*(6′)-Ib-cr, *str*A, *str*B, *aph*(3″)-I/II, *aad*A, *aph*A, *gyr*A, *gyr*B, *sul*1, *sul*2, *dfr*A1, *dfr*A5, *dfr*A7, *dfr*A12, *dfr*A15, *dfr*A16, and *dfr*A17 and *mcr*-1	
	K. pneumoniae	*bla*$_{TEM}$-, *bla*$_{SHV}$, *bla*$_{SHV-2}$, *bla*$_{SHV-11}$, *bla*$_{SHV-27}$, *bla*$_{SHV-28}$, *bla*$_{SHV-52}$, *bla*$_{SHV-61}$, *bla*$_{SHV-83}$, *bla*$_{SHV-98}$, *bla*$_{SHV-108}$, *bla*$_{SHV-148}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-8}$, *bla*$_{CTX-M-14}$, *bla*$_{KPC}$, *bla*$_{NDM-1}$, *bla*$_{oxa-48}$, *bla*$_{NDM-5}$, *tet*(A), *tet*(B), *tet*(D), *aac*(6′)-Ib-cr, *str*A, *str*B, *aph*(3″)-I/II, *oqx*AB, *sul*1, *sul*2, *dfr*A1, *dfr*A5, *dfr*A7, *dfr*A12, *dfr*A15, *dfr*A16, *dfr*A17 and *mgr*B	
Mastitis and Metritis	*T. pyogenes*	*bla*$_{P1}$, *tet(W), orfE, ermB, ermX, dfr2a, aadA2, aadA1* and *aacC*	(Naranjo-Lucena & Slowey, 2023; Rezanejad et al., 2019)
BRD	*M. haemolytica*	*floR, aph3-Ia, tet(H), strA, strB, erm42, ermF, sul2* and *cat2*	(Holschbach et al., 2020)
	P. multocida	*ROB-1, floR, tet(H)*, *aph3-Ia*, *erm42*, *strA*, *strB* and *sul2*	
	H. somni	*floR, aph3-Ia, tet*(H), *strA, strB, MLS*$_b$, *erm42, ermF, sul2* and *dfrA14*	
Calf Diarrhea	*E. coli*	*bla*$_{CTX–M}$, *bla*$_{TEM}$, bla$_{SHV}$, *floR*, *tetB*, *tetA*, *tetD*, *parC*, *gyrA*, *qnrD*, *qnrS*, *strA-B*, *aadAI,* and *sul2*	(Jia et al., 2022)
	Salmonella spp.	*bla*$_{CMY-2}$, *bla*$_{TEM-1}$, *bla*$_{SHV-12}$, *dfrA, aadA1, dfrA1-aadA1, aadA2, dfrA1-sat2-aadA1, qnrS, aac(6)-Ib-cr 15*	(Welsh et al., 2004)

2.1.4.3 Hoof diseases

Lameness has major negative impacts on animal welfare, production and economy of dairy farms, being the third most common cause of culling or premature removal from the herd (Thomsen et al., 2023). Some cases, including digital dermatitis, can be caused by infectious agents, such as bacteria from the *Treponema* genus, including *T. denticola, T. maltophilum, T. medium, T. putidum, T. phagedenis,* and *T. paraluiscuniculi.* Additionally, Kot *et al*. recently isolated *Sphingomonas paucimobilis*, *Ochrobactrum intermedium* I, *Ochrobactrum intermedium* II, *Ochrobactrum gallinifaecis*, and *Actinomyces odontolyticus* for the first time from tissue, pus, blood, and swab samples obtained from the limbs of cattle diagnosed with lameness (Kot et al., 2023).

Lameness can also be associated with Bovine Foot Rot (BFR), an infectious disease of the interdigital skin and subcutaneous tissues of beef and dairy cattle. A plethora of factors related with the host, agent, and the environment are linked to the development of BFR, being *Fusobacterium necrophorum*, *Porphyromonas levii*, and *Prevotella intermedia* the pathogens most commonly associated with this disease (Van Metre, 2017).

2.1.4.3.1 Fusobacterium necrophorum

Fusobacterium necrophorum is an anaerobic Gram-negative bacterium involved in BFR pathogenesis. Early systemic antimicrobial therapy commonly leads to infection resolution, while delayed treatments may result in infection dissemination into deeper structures, such as bone, synovial structures, or ligaments, and are responsible for a worse recovery prognosis (Van Metre, 2017). Intrinsic AMR has not been described regarding this pathogen, however, the resistance genes already reported in isolates from this species and responsible for hoof diseases are summarized in Table 4.

2.1.4.4 Bovine Respiratory Disease

Bovine Respiratory Disease (BRD) can cover a range of respiratory illnesses, ranging from acute fatal respiratory disease to chronic respiratory disease, and consists of a multifactorial condition associated with several causative agents, including bacteria and viruses (Thomsen et al., 2023).The main bacterial species responsible for BRD are *Mannheimia haemolytica*, *Histophilus somni*, *Pasteurella multocida*, and *Mycoplasma bovis.*

A key hurdle in addressing this condition lies in the pathogens' capacity to concurrently infect cells within the respiratory tract (Gandhi et al., 2023).

Despite vaccination being available, it has shown inconsistent efficacy against *M. haemolytica*, *P. multocida*, and *H. somni* infections, making antimicrobials the primary resort for controlling BRD (Gaudino et al., 2022). However, a study from Klima *et al.* (2014) performed with 68

isolates associated with BRD showed that 72% of *M. haemolytica* and 50% of *P. multocida* isolates evaluated were resistant to one or more of the antibiotics tested, which included ampicillin, penicillin, gentamicin, oxytetracycline, tilmicosin, tulathromycin, danofloxacin, enrofloxacin, spectinomycin, florfenicol, neomycin and chlortetracycline. Moreover, 30% of the *M. haemolytica* isolates and 12.5% of the *P. multocida* isolates tested were resistant to more than seven antimicrobial classes, including aminoglycosides, penicillins, fluoroquinolones, lincosamides, macrolides, pleuromutilins, and tetracyclines (Klima et al., 2014; Thomsen et al., 2023).

From all agents responsible for BRD, in this review, we will focus on *M. haemolytica*, *P. multocida,* and *H. somni*. The ARG associated with bacteria responsible for this disease are compiled in Table 4.

2.1.4.4.1 Mannheimia haemolytica

M. haemolytica is a Gram-negative species currently classified based on 12 capsular serotypes (A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, and A17), with A1 and A6 being most frequently associated with respiratory disease in cattle (Gaudino et al., 2022).

From 2008 to 2017, a decline in susceptibility of *M. haemolytica* isolates to tilmicosin, tulathromycin, florfenicol, fluoroquinolones, and gentamicin was observed. (Holschbach et al., 2020).

2.1.4.4.2 Pasteurella multocida

P. multocida, another Gram-negative bacterium, is currently classified into five capsular groups (A to E) and 16 somatic serotypes (1 to 16). In cattle, A:3 is the most common serotype isolated from animals displaying BRD (Gaudino et al., 2022).

Regarding AMR, *P. multocida* has shown low susceptibility to florfenicol, spectinomycin, tetracycline, tilmicosin, and trimethoprim-sulfamethoxazole (Welsh et al., 2004).

2.1.4.4.3 Histophilus somni

H. somni is also a Gram-negative bacterium that affects mainly cattle but also small ruminants, being weaned calves at a higher risk of infection. One of the main problems associated with *H. somni* infections is that the bacteria from this species have the capacity to colonize the lungs of the host and gain access to the bloodstream, causing systemic diseases including encephalitis, myocarditis, and acute septicemia associated with sudden death. Treatment options for *H. somni* infections include the administration of large-spectrum antibiotics, such as florfenicol (Gaudino et al., 2022).

H. somni presents higher resistance to neomycin and sulfadimethoxine and reduced susceptibility to gamithromycin, clindamycin, tylosin, penicillin, spectinomycin, and

oxytetracycline. Additionally, like *M. haemolytica, H. somni* shows low susceptibility to tilmicosin, tulathromycin, and gentamicin (Holschbach et al., 2020).

2.1.4.5 Calf Diarrhea

Similarly to BRD, calf diarrhea is a multifactorial disease associated with several pathogens including viruses, bacteria, and protozoa, with studies reporting an incidence ranging between 5–23% in calves from dairy, beef, and veal production systems (Cho & Yoon, 2014; Wilson et al., 2023; Zhang et al., 2022).

Of the several agents implicated in calf diarrhea, the main ones are bovine rotavirus, bovine coronavirus, bovine viral diarrhea virus, *Salmonella enterica*, *E. coli, Clostridium perfringens*, and *Cryptosporidium parvum*, along with newly emerging enteric pathogens such as bovine *Torovirus* and *Caliciviruses* (bovine Norovirus and Nebovirus) (Cho & Yoon, 2014). However, in this review, we will only focus on the most prevalent pathogens associated with calf diarrhea which control requires antimicrobial use: *Salmonella enterica* and *E. coli* (Zhang et al., 2022). Table 4 summarizes the ARG reported for bacterial species associated with this disease.

2.1.4.5.1*Salmonella* spp.

Salmonella is a Gram-negative, facultative anaerobe and facultative intracellular bacteria. It is a well-known pathogen responsible for gastrointestinal diseases in a wide range of hosts, including humans and cattle. *S. enterica* serovar Typhimurium and *S. enterica* serovar Dublin are the most common causative agents of salmonellosis in cattle, with *S.* Typhimurium being mostly associated with acute diarrheal disease and *S.* Dublin with systemic disease (Cho & Yoon, 2014).

In *Salmonella*, intrinsic antibiotic resistance is mainly associated with its outer membrane, the presence of efflux pumps, and the expression of antibiotic-inactivating enzymes, associated with resistance to vancomycin and fluoroquinolones. Moreover, *Salmonella*'s ability to form biofilms and persister cells play a critical role in their AMR capacity (Zhou et al., 2023). Persister cells are a special subpopulation of bacteria that can transiently tolerate antibiotics by presenting a slow or arrested growth, but that have the ability to resume growth after the removal of antibiotic stressors from the bacterial environment (Fisher et al., 2017).

2.1.4.5.2 Escherichia coli

E. coli was already briefly described above regarding mastitis. Regarding calf diarrhea, the most common *E. coli* strains reported are enterotoxigenic strains (ETEC), namely *E. coli* K99, an ETEC strain that produces the K99 (F5) adhesion antigen, responsible for neonatal diarrhea .

2.1.4.6 Resistance genes

The table 4 summarizes available information regarding antimicrobial resistance genes (ARG) already reported in bacterial isolates responsible for the dairy cattle diseases mentioned above.

2.1.5 AMR spread sources

The possible relationship between antibiotic usage and AMR is a main concern of the scientific community and governmental authorities, especially from a One-Health perspective. Despite no direct association between AMU, AMR, and interspecies resistance transmission has been consistently reported, most studies support that a combined approach to tackle AMR is the most promising solution. Therefore, the combination of efforts to control and reduce the use of antimicrobials in human and veterinary medicine could potentially decrease the overall AMR resistance, which reinforces the importance of the One Health approach (de Greeff et al., 2022; Richardson et al., 2018).

Resistance spread has also been associated with biological excreta such as milk, urine, and feces. Thus, these sources can not only contribute to the dissemination of resistant bacteria but also of ARG and antibiotic residues (Oliver et al., 2020).

Taking this into account, in the following topics we will focus on the different ways by which antimicrobial residues, resistant bacteria, and resistance genes can be disseminated between animals, the environment, and humans, as well as on possible strategies to control this spread.

2.1.5.1 Waste Milk

Waste milk (WM) comprises all milk produced that is not marketable, which includes colostral milk, milk from cows with clinical mastitis, milk collected during the withholding period after administration of veterinary drugs to the animals, milk with a high somatic cell count, and milk that exceeds the milk quota and cannot be marketed. However, to repurpose WM, farmers often feed it to calves, avoiding the high costs of milk replacers (Aust et al., 2013; Ma et al., 2022; Penati et al., 2021). The importance of the different antimicrobials that may be present in WM differs from a public health perspective, and the risk associated with them depends on numerous factors, including the type of drug administered to the animals, dosage, timing of administration relative to milking, and route of administration (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2017).

There are several studies available on bacterial isolates recovered from both raw milk and WM samples (Balemi et al., 2021; Endimiani et al., 2012; Frey et al., 2013; G. Liu et al., 2020; Tark et al., 2017; Yu et al., 2020), portraying how milk can be a reservoir for AMR bacteria and ARG (Balemi et al., 2021; Bonardi et al., 2023; Brunton et al., 2012; EFSA Panel on Biological Hazards (BIOHAZ) et al., 2017; Eldesoukey et al., 2022; Fergestad et al., 2021;

Gosselin et al., 2022; Hamel et al., 2020; S. Huang et al., 2022; Olasoju et al., 2021; Stevens et al., 2019). Aminoglycoside resistance genes *ant*(6)-Ia (conferring resistance to streptomycin), *aac*(6′)-Ie–*aph*(2′)-Ia (to gentamicin, tobramycin, amikacin), or *aph*(3′)-III (to kanamycin, neomycin, amikacin, gentamicin B, paromomycin) were found in NAS from bovine mastitic milk in Switzerland (Frey et al., 2013). Additionally, the plasmid-borne gene bla_{CMY-2} was detected in *E. coli* isolates from bovine milk in Switzerland, Thailand, South Korea, and Lebanon (Abboud et al., 2021; Endimiani et al., 2012; Hinthong et al., 2017; Tark et al., 2017). The bla_{NDM-1} gene has been detected in *E. coli* isolated from the milk of cattle with clinical or subclinical mastitis in India, whereas in Pakistan, cows' milk samples have been reported as containing *K. pneumoniae* carrying both the bla_{NDM-1} and the bla_{oxa-48} genes . Also, the bla_{NDM-5} gene has been identified in *E. coli* and *K. pneumoniae* from milk samples from Algeria and China (Yaici et al., 2016; Yu et al., 2020). Lastly, the presence of *rmt*B in *E. coli* and *K. pneumoniae* isolates obtained from milk samples in China is one of the very few reports indicating the presence of this gene in bacteria from animal-derived products (Yu et al., 2020). The presence of these AMR bacteria in WM indicates that this product can potentially be responsible for the dissemination of these strains to soil, water, or calves through feeding. A study from 2013 by Aust *et al.* (2013) on bacterial isolates from calves fed with bulk milk (BM) and raw or pasteurized WM showed that the proportion of resistant *E. coli* isolates, including isolates resistant to cefotaxime, nalidixic acid, and sulfamethoxazole & trimethoprim, was significantly higher in calves fed with raw or pasteurized WM in comparison with calves fed with BM. These authors support that pasteurized WM from cows not treated with antimicrobials could be an acceptable feed option for young calves (Aust et al., 2013).

Later in 2017, Maynou *et al.* (2017) studied the antimicrobial resistance patterns of fecal *E. coli* and nasal *Pasteurella multocida* isolates from calves fed either with milk replacer (MR) or WM. These authors reported a higher number of *E. coli* isolates resistant to enrofloxacin, florfenicol, and streptomycin, as well as multidrug-resistant *E. coli* phenotypes in the feces of calves fed with WM (Maynou, Bach, et al., 2017). In further studies, these authors reported that feeding WM to animals increases the prevalence of pathogenic microbiota resistant to antimicrobials (Maynou, Bach, et al., 2017; Maynou, Migura-Garcia, et al., 2017). Additionally, they concluded that WM produced by cows treated with β-lactam antimicrobials contained drug residues in concentrations high enough for selecting resistant *E. coli* in the calf gut. Moreover, the presence of florfenicol-resistant *E. coli* in WM-fed calves to which this antibiotic was never administered, may suggest that the antimicrobial residues present in milk may exert selective pressure to the gut microbiota, leading to the development of bacterial resistance to other antimicrobials. However, the presence of high levels of both phenotypic and genotypic resistance to tetracycline and aminoglycosides in calves independently of feeding regimens impairs the establishment of a direct relation between WM feed and AMR transmission

(Maynou, Migura-Garcia, et al., 2017).

Following the inconsistent findings on the relation between WM feeding and AMR development, the European Commission (EC) requested EFSA to deliver a scientific opinion on the risk for the development of AMR due to feeding of calves with milk containing residues of antibiotics. In their report, they concluded that the risk of AMR and ARG spread through WM was a real threat (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2017).

Later in 2021, Firth *et al.* (2021) reviewed the subject, concluding that the available studies from 2016 to 2020 were mostly limited to *E. coli,* which is one of the most common bacteria in dairy farms, pointing to the need of addressing other bacterial species for a more comprehensive evaluation of WM feeding effects(Firth et al., 2021).

Another concern regarding feeding with WM is the duration of bacterial shedding. While WM appears to increase the excretion of AMR bacteria by dairy calves, Firth *et al*. refer that such shedding is frequently temporary and transient, appearing not to pose a long-term threat. Moreover, authors state that, despite changes in the calves' microbiome following WM feeding being commonly reported in the literature, there are no consistent results available on whether the effect that these changes have on calf health are indeed positive or negative (Firth et al., 2021).

Another review from 2022 by Ma *et al.* (2022) points out the positive advantages of WM feeding to the farm economy and to the growth and performance of dairy calves. However, authors also emphasize the risks related with feeding untreated WM to calves, mostly associated with direct bacterial transmission from cow to milk, and also with poor hygiene practices during milking, transportation, and milk storage (Ma et al., 2022).

In general, the existing research on WM remains inconclusive regarding the existence of a direct link between WM use in calf feeding and AMR dissemination. This uncertainty is largely attributed to limited sample sizes and variations in testing methods performed in the different studies; as such, there is a crucial need for globally standardized research strategies for addressing this issue.

2.1.5.2 Manure

The use of manure as a soil amendment is a common agricultural practice worldwide, not only due to its richness in nutrients and organic matter but also because it is a cost-effective way of disposing of liquid manure (slurry), especially considering that a single dairy cow can produce an average of 54 kg of slurry per day (De Liguoro et al., 2003; Zalewska et al., 2021). Additionally, manure can enhance crop growth and development by actively cycling chemicals such as phosphorus and nitrogen. However, like other products, if it is misused or overused, it may have deleterious effects, including the accumulation of these compounds in the farm environment. Another possible side effect of manure application to soil is the increased risk of

environmental contamination with antibiotic residues, antibiotic-resistant bacteria (ARB), and ARG (Oliver et al., 2020; Zalewska et al., 2021). However, several factors influence the potential risk associated with manure application to soil, such as time and conditions of manure storage, contaminants characteristics, application method, time period of UV exposure after soil application, and weather conditions during and after manure application (Fangueiro et al., 2021).

For instance, the effect of antibiotic residues and their persistence in the farm environment depends on the soil's physicochemical characteristics, as well as on the climate. In sandy soils, the leaching of antibiotics is higher than in clay or silty soils. Additionally, the antibiotics themselves have different capabilities of penetrating the different soil layers, with sulfamethazine and erythromycin easily reaching the deeper layers and even groundwater, posing an increased risk of further spreading antibiotic residues (Zalewska et al., 2021).

Moreover, some antibiotics, such as tetracyclines and quinolones, are able to adhere to soil particles, thus accumulating in the soil, changing its natural microbiome, and promoting ARG maintenance. For instance, the full biotransformation of oxytetracycline in cattle manure applied to soil takes up to 150 days; however, it is not clear if these antibiotics are still active after manure storage (Zalewska et al., 2021).

Cattle manure is considered a reservoir for ARG, with associated resistomes, which comprise all antibiotic-resistance genes found in a given environment, varying from herd to herd (Oliver et al., 2020). Moreover, Gram-negative bacteria are very prevalent in manure, which may contribute to increasing the flow of mobile genetic elements like ARG (Buta-Hubeny et al., 2022). In dairy cow manure, ARG can coexist extracellularly on plasmids, as well as in transposable elements and bacteriophages. Recent research on ARG associated with *E. coli* has demonstrated herd-level resistome diversity, sometimes with variations in ARG encoding for resistance to the same antibiotic (Qian et al., 2018). According to a sequencing effort of 160 antibiotic-resistant *E. coli* isolates and assessment of 28 fecal metagenomes, tetracycline resistance appears to be the most prevalent, corresponding to 61% of all detected ARG (Haley et al., 2017). Similar results were found by Pereira *et al*. (2021) who reported that the most frequent ARGs in cattle manure are *tet*, *sul*, and *erm* (Pereira et al., 2021). According to a recent study by Buta-Hubeny *et al*. (2022), fresh bovine manure applied as fertilizer is colonized mostly by Actinobacteria (29%), Bacteroidetes (16%) and Proteobacteria (9%), presenting a predominance of genes associated with multidrug resistance, such as *cfr* (59%), followed by genes encoding for resistance to macrolides, lincosamides, and streptogramins (9%), bacitracin (5%), fosmidomycin (5%), aminoglycosides (4%), vancomycin (4%), tetracyclines (4%) and sulfonamides (3%). Despite the high number of resistances found in bacteria from cattle manure, it is important to note that the concentrations of these ARG did not increase significantly after its use as soil fertilizer, nor lead to considerable changes in the

structure of the soil resistome, in contrast with was observed for poultry manure, which was found to contribute for an increased diversity of ARGs in soil (Buta-Hubeny et al., 2022).
Besides antibiotics residues, ARB and ARG can also spread from soil to plants, with studies detecting *sul*2, *erm*F, $bla_{PSE,}$ and bla_{OXA-2O} genes in plants grown in dairy manure-amended soil (Zalewska et al., 2021). Common soil bacteria can transfer ARG to plants through the root endophytes, being able to survive in these structures. In fact, Solomone *et al*. (2002) reported the transmission of *E. coli* O157:H7 from manure-amended soil and water irrigation to lettuce, colonizing the vegetable through the root, after which spread to the edible parts (Solomon et al., 2002).
In conclusion, several studies support that the application of manure containing ARB and ARGs increases the risk of ARB and ARG transmission to the environment. and therefore to humans. (Buta-Hubeny et al., 2022; Chen et al., 2018, 2019; Duan et al., 2019; Hu et al., 2016; Keenum et al., 2021; Laconi et al., 2021; Li et al., 2020; Menz et al., 2019; Oliver et al., 2020; Peng et al., 2017; Pu et al., 2019; Qian et al., 2018; Ruuskanen et al., 2016; Zalewska et al., 2021). However, due to the manure variability, several alternatives will be discussed later.

2.1.6 Strategies to Fight Back Against AMR

To successfully tackle AMR, a combined approach is of paramount importance, focusing not only on the source of resistance but also on its dissemination routes through the different environments. Therefore, it should focus on three main pillars: controlling antibiotic usage; finding alternatives to conventional antimicrobials; and promoting the decontamination of residues and resistance genes.

2.1.6.1 Monitoring and Control of AMU and AMR

2.1.6.1.1 Legislations and Programs

In this review, we are going to mainly focus on the legislation and programs aiming to control antibiotic use in place, as well as on the procedures that can be applied in the field to improve the selection of antibiotics to be administered.
The World Health Organization (WHO) (2015) paved the road for the fight against AMR with the publication, in 2015, of a Global Action Plan on Antimicrobial Resistance. Through the One Health project, the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (WOAH) worked together with WHO to put into practice pertinent strategies to fight AMR worldwide. To address the global threat that AMR represents, this partnership promotes the development of efforts in the human and animal health settings, as well as in the environmental sector. Strengthening the knowledge and evidence basis through surveillance and research is one of the goals of this action plan (World Health Organization,

2015).

With Directive 2003/99/EC and Decision 2013/652/EU, which has since been reinforced by the Decision (EU) 2020/1729, the EU established the requirement for AMR surveillance in zoonotic (*Salmonella* and *Campylobacter*) and indicator bacteria (*E. coli*) from healthy food-producing animals (cattle, poultry, and pigs). Although a harmonized surveillance of veterinary clinical isolates is still not performed in the EU, several European countries, such as the Netherlands, Sweden, Denmark, Germany, France, United Kingdom, and Portugal, are surveying AMR in veterinary clinical isolates as part of their AMR National Plans for some years now (Naranjo-Lucena & Slowey, 2023). International harmonization methodologies for the investigation of veterinary AMR are of paramount importance since there are still major variations in the methods employed by different laboratories aiming at antimicrobial susceptibility testing (disk diffusion, minimal inhibitory concentration determination) and in the standards followed for interpretation (Clinical & Laboratory Standards Institute, CLSI; European Committee on Antimicrobial Susceptibility Testing, EUCAST; *Le Comité de l'Antibiogramme de la Société Française de Microbiologie*, CA-SFM; Animal Health and Veterinary Laboratories Agency, AHVLA), making the global comparison of results challenging (Mader et al., 2022).

Since 2017, the EU Joint Action on Antimicrobial Resistance and Healthcare-Associated Infections (EUJAMRAI) (Mader et al., 2022), published the best practices and policies for the proper implementation of national plans. The European Antimicrobial Resistance Surveillance network in Veterinary Medicine (EARS-Vet), which brings together professionals working in AMR surveillance in animals within the EU in an effort to establish best practices and standardize and harmonize antimicrobial susceptibility testing in the veterinary field, was born out of this project (Mader et al., 2022). While a database of this magnitude can take years to implement, user-fed databases, such as resistancebank.org, offer complementary data with known limitations, primarily regarding the willingness of independent users to share their information, as well as the lack of results harmonization (Criscuolo et al., 2021).

The EU Commission requested the European Medicines Agency (EMA) and the Antimicrobial Advice Ad Hoc Expert Group (AMEG) to provide scientific advice on the impact of antibiotics administration to animals on both public and animal health, and to propose steps to mitigate any potential danger to humans. In order to assist veterinarians in making treatment decisions, EMA has categorized antibiotics according to their risk of resistance development (EMA, 2018).

Moreover, Regulation (EU) 2019/6 on veterinary pharmaceutical goods and Regulation (EU) 2019/4 on medicated feed, established the need to restrict the use of antibiotics to prevent the emergence of new resistant strains. Through these new regulations and the reservation of specific antimicrobials solely for human use, this new legislation seeks to decrease the use of

antibiotics in the field of animal health. This circumstance is expected to motivate veterinarians to ask for an antimicrobial susceptibility test before prescribing critical compounds, while also supporting antibiotic selection and maximizing therapeutic effectiveness (Naranjo-Lucena & Slowey, 2023).

However, in low-and-middle-income countries from Asia, Africa, and South America, the absence of systematic surveillance systems and the lack of legislation limits the possibility of establishing global actions to control AMR. For instance, in 2016, China implemented a national pilot program to reduce unnecessary antimicrobial use, while in India the action plan for AMU was established in 2012, and is still being implemented (Holmes & Sharland, 2013; Lim & Grohn, 2021; Van Boeckel et al., 2019). However, in the new FAO action plan for 2021-2025, an incentive is predicted for developing countries to enforce the Implementation of AMU control strategies (Food and Agriculture Organization of the United Nations, 2021; Holmes & Sharland, 2013).

2.1.6.1.2 Selective Antimicrobial Treatment

Besides the current legislation that limits which antibiotics can be used, the identification of pathogens responsible for disease in dairy farm animals and the characterization of their antibiotic susceptibility profile would help reduce antibiotic use in these settings.

Regarding antimicrobial use in dairy farms, most literature points to dry cow therapy and clinical mastitis as the main reasons for antibiotic administration (Farrell et al., 2023; McCubbin et al., 2022).

Blanket dry cow therapy (BDCT) consists of a treatment with a long-acting intramammary antibiotic infusion applied to all cows between lactation cycles, intending to treat existing infections and prevent new ones. However, BDCT implies administering antibiotics disregarding the animals' infection status or disease incidence risk during the dry period (Farrell et al., 2023; McCubbin et al., 2022). Due to the detection of genes conferring resistance to β-lactam antibiotics, third-generation cephalosporins, and aminoglycosides in animals subjected to dry-off therapy, the European Commission prohibited the prophylactic use of antibiotics, including the routine antibiotic treatment of all quarters from cows at drying-off regardless of their infection status. However, in other parts of the world with no available regulation on this subject, these treatments are still in place, posing a risk for resistance dissemination and consequently a danger to animal and human health (de Jong et al., 2023).

Selective dry cow therapy (SDCT) consists of only applying antibiotic treatment based on the risk of the animals developing an intramammary infection (IMI) during the dry period, as this is an important risk factor for mastitis in the early subsequent lactation. The selection of which cases to treat, instead of indiscriminately administering antibiotics, has largely reduced the use of antibiotics for mastitis treatment (de Jong et al., 2023). SDCT started being applied in

Norden Europe since the 70s with favorable results. For instance, in Norway, the clinical cases of mastitis decreased 73% between 1994 and 2018 (Hommels et al., 2021; Rajala-Schultz et al., 2021).

However, several studies found a concerning relation between SDCT and an increased risk of developing IMI in the subsequent lactation. Winder *et al.* (2019) reviewed several of these reports, concluding that SDCT was associated with a higher risk of IMI at calving in comparison to BDCT (RR = 1.34, 95% CI = 1.13, 1.16). Still, this systematic review revealed a trend for the combination of teat sealants with SDCT not being associated with an increased risk of IMI. Moreover, this review emphasized the possibility that the variable criteria applied in the selection method may affect the association of SDCT with the risk of IMI (Winder et al., 2019). McCubbin *et al*. (2022) also reviewed the implications of SDCT for Dairy Farms, and their conclusion was aligned with the work by Winder *et al.* (2019) regarding the possibility of complementing this treatment with teat sealants (McCubbin et al., 2022).

Nevertheless, a study by Rowe *et al.* (2020) proposed that SDCT program failures may be attributed to insufficient diagnostic screening strategies to detect IMI, to the lack of a teat sealant to protect against new IMI during the dry period, or to both. To overcome the limitation of SDCT, this author suggested applying culture-guided (Cult-SDCT) and algorithm-guided SDCT (Alg-SDCT) programs to increase the selection sensitivity while using teat sealants. While Alg-SDCT allows to compare several criteria and protocols applied in the past, with statistical analysis results being summarized as low or high risk, Cult-SDCT is based on microbiological testing to detect and identify the pathogens responsible for the infection. Through a series of studies, Rowe *et al.* (2020,2021,2023) concluded that both Cult-SDCT and Alg-SDCT could still contribute to substantially reduce AMU at dry-off, without negatively affecting IMI risk, or the milk yields (Rowe et al., 2023; Rowe et al., 2020, 2021).

Additionally, culture testing applied to the direct treatment of clinical mastitis could also contribute to reducing the AMU by identifying the causative agent. While samples processing by a laboratory can take more than 24h to provide results, commercially available on-farm rapid tests can identify the causal agent quickly, contributing to the establishment of a targeted therapy. The first on-farm culture systems allowed the categorization of samples as presenting Gram-positive and Gram-negative microorganisms or no growth, within 24 to 32 h, with most of them allowing the identification of Gram-positive bacteria with a sensitivity ranging from 59% to 98%, and a specificity ranging from 48% to 97% (Ganda et al., 2016; Tommasoni et al., 2023). More recent systems can presumptively differentiate between bacterial species and/or groups according to the color of the bacterial colonies obtained. According to a review by Tommasoni *et al.* (2023), these chromogenic media can be highly sensible and specific. For instance, CHROMagar ™ presents 100% sensibility and 99.8%, specificity for the identification of *S. aureus* in clinical mastitis milk samples, being also adequate for the

identification of *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. in subclinical mastitis. However, the observers' experience in interpreting results is crucial for the success of these systems and for the implementation of the right treatment protocol (Tommasoni et al., 2023).

When comparing the application of culture testing of samples from animals with clinical mastitis subjected to BDCT, a review by Jong *et al.* (2023)concluded that selective treatment of non-severe clinical mastitis can be adopted to successfully reduce AMU without negatively impacting udder health (de Jong et al., 2023).

2.1.6.2 Alternatives to Antibiotics

Innovative antimicrobial approaches, including antimicrobial peptides, phage therapies, and even nanoparticles, have been thoroughly investigated by the scientific community aiming to reduce antibiotic use, with new possible solutions and theories emerging every day. This review will focus on specific reports regarding the use of alternative antimicrobial strategies in dairy farms.

2.1.6.2.1 Nanoparticles

With the increasing development of nanotechnology, nanoparticles (NPs) have become a promising asset for several uses, including as targeted drug delivery systems, diagnostic systems, noninvasive imaging technologies, and antimicrobial compounds. Their unique physicochemical properties, like resistance, durability, performance, and flexibility, paired with action mechanisms completely different from the ones of traditional antibiotics, make them good candidates for substitute therapeutics (Fatima et al., 2021). Their use has been more explored regarding human medicine, still, there are already a few studies available directed towards animal health (Fatima et al., 2021; Gurunathan et al., 2018; Kalińska et al., 2019, 2023; Kot et al., 2023; Tripathi & Goshisht, 2022; Vasiliev et al., 2023). The applications and action mechanisms of several NPs with potential use in dairy farms are summarized in Table 5.

Table 5 - Summary of Nanoparticles with potential use in dairy farms, focusing on target microorganisms and action mechanisms.

Nanoparticles	Target Microorganisms	Action Mechanism	References
Silver (Ag)	*P. aeruginosa, V. cholerae, K. pneumoniae, S. aureus, E. faecium, S. epidermidis,* and *T. pyogenes.*	Adhesion to the bacterial cell surface causing membrane injury and affecting transport activity; Penetration of AgNPs inside the microbial cells, where interaction with cellular organelles and biomolecules damages the respective cellular machinery; Trigger an increase in reactive oxygen species inside the microbial cells which in turn cause cell damage; Modulation of cellular signal transduction pathway and finally cause cell death.	(Fatima et al., 2021; Gurunathan et al., 2018; Tripathi & Goshisht, 2022)
Copper oxide (CuO)	*B. subtilis, S. aureus, E. coli, E. faecalis, E. cloace, S. agalactiae, C. albicans, K. pneumoniae, P. aeruginosa, Propionibacterium acnes,* and *Salmonella* Typhi.	Reduce bacteria attachment to the cell wall; Disrupt the biochemical processes inside bacterial cells.	(Fatima et al., 2021; Kalińska et al., 2019)
Gold (Au)	Methicillin-resistant *S. aureus, S. paucimobilis, O. gallinifaecis,* and *A. odontolyticus.*	Generate holes in the cell wall; Binds to the DNA and inhibits the transcription process.	(Fatima et al., 2021; Kot et al., 2023)
Silver-Copper (AgCu)	*S. aureus, S. epidermidis, E. coli, E. faecalis, E. cloacae, S. agalactiae, Candida albicans, S. paucimobilis, O. intermedium I, O. intermedium II, O. gallinifaecis* and *A. odontolyticus.*	Same mechanism as AgNPs and CuONP separately with: • Better dissolution of Ag in the presence of Cu ions due to oxidation in the redox reaction • Production of more antibacterial Cu ions during the same redox reaction • Less favorable binding of Ag ions to medium proteins in the presence of Cu ions	(Fatima et al., 2021; Kalińska et al., 2019; Kot et al., 2023; Vasiliev et al., 2023)

For instance, Gurunathan *et al.* (2018) tested the use of silver nanoparticles (AgNPs) as an alternative therapeutic for the treatment of uterine infections in dairy cattle, by synthesizing AgNPs using apigenin and testing their *in vitro* inhibitory potential towards *Prevotella melaninogenica* and *T. pyogenes* isolates obtained from uterine samples of cows. AgNPs are known for their antimicrobial potential towards drug-resistant bacteria (Gurunathan et al., 2018). The silver ions (Ag+) discharged from AgNPs enhance bacterial membrane permeability and produce reactive oxygen species (ROS) that damage cell walls. Besides, the antibacterial activity of AgNPs is also linked to their penetration inside the bacterial cell, followed by the destruction of intracellular structures (ribosomes, mitochondria, and vacuoles) and biomolecules (DNA, lipids, and proteins), as well as by modulation of intracellular signals transduction pathways (Tripathi & Goshisht, 2022). The AgNPs synthesized by Gurunathan *et al.* (2018) exhibited significant antibacterial and anti-biofilm activity against the tested isolates *in vitro* (Gurunathan et al., 2018). However, they still needed to be tested *in vivo*, since more recent *in vitro* studies reported that AgNPs present cytotoxic effects towards human cell lines, specifically those with sizes ≤10 nm. Besides, it has also been shown that AgNPs can cross the blood-brain barrier of mice, causing neurotoxicity, and neuronal death. Additionally, the accumulation of AgNPs in several organs of mice/rats was also observed, therefore raising concerns about the safety of the application of these NPs in mammals with therapeutic purposes (Tripathi & Goshisht, 2022).

Kalinska *et al.* (2019) tested AgNPs, copper NPs (CuNPs), and combined silver and copper NPs (AgCuNPs) using both human and bovine cells, aiming to establish an alternative treatment for mastitis (Kalińska et al., 2019). Authors observed that these NPs, especially in lower concentrations, not only presented a positive antimicrobial effect against *Enterococcus faecalis*, *E. coli*, *S. aureus*, *Enterobacter cloacae*, *S. agalactiae*, and *Candida albicans* but also appeared to be safe for human and bovine use since no toxic effects were observed neither towards a bovine mammary epithelial cell line (BME-UV1) or a human mammary epithelial cell line (HMEC) (Kalińska et al., 2019). Furthermore, this research group is aiming to develop a disinfectant to be used in the milking routine of dairy cows. In their latest publication from 2023, they combined NPs with cosmetic substrates (collagen + elastin, glycerin, sorbitol, propylene glycol, d-panthenol, vitamin C, sodium lactate, urea, and marigold flower extract) to protect the udder skin. They concluded that the combination of NPs and cosmetic substrates could be effective in preventing mastitis by *S. aureus* and *E. coli*, but also that, on their own, propyleneglycol and vitamin C can reduce bacteria presence by 35–50% (Kalińska et al., 2023).

Kot *et al.* (2023) also investigated NPs potential as an alternative treatment for digital dermatitis, by studying the properties of AgNPs, CuNPs, gold NPs (AuNPs), platinum NPs (PtNPs), iron NPs (FeNPs) against pathogens isolated from cows suffering from hoof

diseases: *S. paucimobilis, O. intermedium I, O. intermedium II, O. gallinifaecis,* and *A. odontolyticus*. Similarly to the studies referred above, AgNPs, AuNPs, and CuNPs exhibited the strongest antibacterial properties, only surpassed by the complex AgCuNPs, while PtNPs and (FeNPs) showed very weak antibacterial activity, even promoting bacterial growth in certain cases (Kot et al., 2023)

Based on these studies, it is evident that NPs, particularly AgCuNPs, seem to be a promising and viable alternative to antibiotics. Nevertheless, conducting additional toxicity studies using different cell lines and models is imperative to ensure its safety for a broader application.

2.1.6.2.2 Antimicrobial peptides - Nisin

Antimicrobial peptides (AMPs) are short proteins with 5 to 100 amino acids, produced by all living organisms, from prokaryotes to eukaryotes. These peptides play a vital role in innate immunity against a range of pathogens, including bacteria, both Gram-positive and Gram-negative, viruses, fungi, and parasites, and even present anticancer activities (Barman et al., 2023; Mazurkiewicz-Pisarek et al., 2023). They are very promising antimicrobials due to their broad-spectrum activity, higher efficiency against various bacterial strains, and lesser tendency for microbial resistance development (Barman et al., 2023).

Their discovery is attributed to Rene Dubos, associated with the isolation of gramicidin from a soil *Bacillus* strain in 1939, which protected mice from pneumococcal infection (Mazurkiewicz-Pisarek et al., 2023). According to the AMP Database's latest update, there are 3569 peptides currently classified as AMP, most of which are produced by animals, followed by bacteria, plants, fungi, and archaea (Wang et al., 2016).

Of those AMP, one is particularly promising for use in dairy cattle. Nisin, a bacterial peptide with 34 amino acids produced by *Lactococcus lactis*, was the first bacteriocin approved by the WHO, FAO, and FDA for use as a food additive to control microorganisms in several food products (Barman et al., 2023; Cao et al., 2007; Wang et al., 2016).

In 1992, Sears *et al.* (1992) suggested nisin application as an effective compound against mastitis, including it as an active ingredient in a teat-dipping product to prevent mastitis (Sears et al., 1992).

In 2007, Cao *et al*. (2007) evaluated the efficacy of a nisin-based formulation for intramammary infusion to be applied in the treatment of clinical mastitis. This study also evaluated the presence of pathogens and nisin residues in milk samples. The authors observed a similar cure rate promoted by nisin and by the control antibiotic treatment (gentamicin), including a bacteriological cure rate of 60.8% and 44.6% for nisin and gentamicin, respectively, and a clinical cure rate of 90.2% for nisin and 91.2% for gentamicin. Moreover, regarding the presence of pathogens in milk samples, more specifically *S. agalactiae* and *S. aureus*, nisin was more effective in their elimination than gentamicin (54.5% vs 33.3%). Finally, nisin was

absent in milk samples after 12h of intramammary infusion (Cao et al., 2007).

Moreover, Bennet *et al.* (2022) reported that nisin can also be effectively applied as a teat-dipping agent to prevent intramammary infections and control mastitis, as its use allowed to control the proliferation of pathogens and reduce bacterial load on teat skin. The authors evaluated the efficacy of bacteriocin-based teat formulas, including bactofencin A, nisin, and reuterin, applied alone or in combination. They observed that the combined application of nisin, bactofencin, and reuterin produced a higher reduction of staphylococci, streptococci, and total bacteria counts, showing that bacteriocins could be considered a good alternative to be used as a teat disinfectant when compared with the iodine positive control (Bennett et al., 2022).

Besides being effective in inhibiting S. *aureus* growth, as reported by Cao *et al.* (2007) nisin also has the ability to impair biofilm formation by *S. aureus* and to reduce the density of established biofilms by methicillin-resistant *S. pseudintermedius*. Nisin also has a protective action against *E. coli* infection by enhancing host immunity, namely by downregulating the release of inflammatory factors, therefore exerting anti-inflammatory activity. However, little is known about the anti-inflammatory mechanisms of nisin as well as its *in vivo* cytotoxicity (Cao et al., 2007; Mazurkiewicz-Pisarek et al., 2023; Sears et al., 1992).

Huang *et al*. (2022) conducted a study using nisin Z, a wild-type variant of nisin, aiming to understand its anti-inflammatory effect on mice and human cell lines. Results showed that nisin Z alleviates inflammation and reduces the release of inflammatory cytokines (IL-6, IL-1β), in both human cell lines and mastitis mice models. Moreover, nisin Z also decreased inflammation by promoting the blood-milk barrier in mastitis mice. Despite these promising results, further studies are required to support the *in vivo* use of this antimicrobial peptide in bovines (F. Huang et al., 2022).

Despite nisin potential, studies revealed that some *Streptococcus* strains may present a gene cluster encoding for a nisin resistance protein (NSR) and an ABC transporter, NsrFP, which confer resistance to nisin. NSR can degrade nisin by cleaving the peptide bond between MeLan28 in ring E and serine at position 29. After cleavage, the resulting molecule has significantly lower bactericidal efficacy and reduced affinity for cell membranes. However, this antimicrobial peptide can be modified through genetic engineering, aiming to produce molecules resistant to NRS action. For example, by replacing serine 29 and isoleucine 30 with proline and valine, respectively, a nisin derivative is obtained, Nisin PV, with enhanced resistance to proteolytic cleavage by NSR (Assoni et al., 2020; Field et al., 2016; F. Huang et al., 2022; Khosa et al., 2013; Perez-Mercado et al., 2019). Besides having improved resistance to cleavage, a study by Pérez-Ibarreche *et al*. (2021) comparing the efficacy of nisin A (wild type) versus nisin PV regarding biofilms inhibition and eradication, also showed that the efficacy of nisin PV far exceeded that of nisin A.

2.1.6.2.3 Phage therapy

Bacteriophages are bacterial viruses that attach, invade, and multiply within their hosts, ultimately leading to bacterial lysis (Mohammadian et al., 2022). Due to their high specificity, phages are a promising alternative approach to substitute or complement the action of conventional antimicrobials. By acting exclusively in their host cells, they do not affect the host's normal microbiota, therefore preventing bacterial dysbiosis and subsequent infections (Mohammadian et al., 2022; Titze et al., 2020). Moreover, the high discovery rate of new phages and the possibility to combine different phages for a higher action spectrum and a reduced risk of resistance development, also support their use as antibiotic substitutes (Saitoh & Shibayama, 2016).

Mohamadiam *et al*. (2022) tested two phages from the Podoviridae family, *Staphylococcus* phage M8 and *Staphylococcus* phage B4, obtained from dairy farm sewage, with specific lytic activity against *S. aureus* isolates. Despite *Staphylococcus* phage M8 showing better antimicrobial results, both phages kept their lytic activity in milk, reducing the *S. aureus* population in spiked milk by about 3 logs after 8h of incubation, supporting their future potential application as biological control agents, alone or in combination (Mohammadian et al., 2022). However, it is important to refer that the use of phages as therapeutic alternatives has some limitations, such as stability, durability, and the onset of immunological host responses, which must be addressed before its wide application *in vivo* (Teklemariam et al., 2023).

2.1.6.3 Decontamination/Remediation

2.1.6.3.1 Milk

There are limited solutions for the removal of antibiotic residues from milk, however, β-lactamases could be an option for the removal of penicillins from this type of substrate. In fact, a study by Li *et al.* (2014) targeting penicillin G, penicillin V, and ampicillin residues present in milk showed that β-lactamases can effectively degrade penicillins; however, they cannot be used for its removal. Still, for future studies, the application of β-lactamases in conjugation with other targeting techniques may be a valid approach (Li et al., 2014).

Moreover, studies focusing on other liquid substrates, such as contaminated water, could serve as a benchmark for assessing the effectiveness of methods aiming at the removal of antibiotics from milk. A study by Saitoh *et al.* (2016) described the use of modified clay minerals, namely organoclay, as a promising alternative sorption method. Authors showed that hydrophobic organic pollutants and polar and ionizable compounds present in water, including penicillin G, nafcillin, cefazolin, cefotaxime, and oxacillin, are incorporated into surfactant aggregates formed between the layers of clay minerals (Saitoh & Shibayama,

2016). In this study, they tested the sorbent potential of Didodecyldimethylammonium bromide (DDAB)-montmorill-onite (MT) organoclay, observing promising results in the removal of β-lactam antibiotics from water and also regarding its eco-friendly degradation (Saitoh & Shibayama, 2016). Due to their successful application in water samples, these clay minerals could also be potentially tested in milk samples.

Recently, Hemmati *et al.* (2023) reviewed the use of molecular imprinting polymers (MIPs) for the removal of antibiotics and other residues from milk samples (Hemmati et al., 2023). MIPs consist of synthetic materials containing specific recognition sites complementary to the target molecules. Due to their high selectivity, MIPs possess the advantage of allowing the extraction of specific chemical contaminants, without the interference caused by other constituents present in the milk matrix. Moreover, MIPs can be tailored to detect various chemical pollutants, both organic and inorganic, and to enhance their affinity towards particular chemical components (Hemmati et al., 2023).

The potential use of MIPs for the removal of milk residues allows the selective isolation of contaminants, having the advantage of being reusable, thereby mitigating the expenses and ecological consequences associated with the extraction procedure. These molecules have already been used in milk samples to isolate and purify milk proteins, such as casein and whey, and to detect milk allergens. Regarding the subtraction of antibiotic residues, including ampicillin, amoxicillin, oxacillin, and penicillin G, they seem to present a higher selective ability of rebinding to ampicillin (Hemmati et al., 2023).

Despite MIP's efficiency and specificity, incomplete template removal and challenges in the scale-up production of MIPs have hampered their commercialization. Therefore, more studies are needed to improve the properties of MIPs as sorbents for commercial applications. Despite only being tested for the detection and removal of small molecules so far, the evaluation of their efficacy towards larger ones, such as bacterial toxins, would be an interesting approach for future studies (Hemmati et al., 2023).

MIPCs (molecularly imprinted polymer-coated stainless-steel sheets) are another promising alternative for fluoroquinolone detection in complex samples, such as milk. However, at present, the application of these compounds is still limited to the detection, and not to the removal, of fluoroquinolones from milk and other aqueous solutions (Hemmati et al., 2023).

2.1.6.3.2 Manure

There are an increasing number of available options for manure pretreatment, aiming to mitigate the threat of soil contamination with AMR bacteria and antibiotic residues originating from the application of untreated manure to the soil. Manure storing is the most common and cost-effective treatment applied prior to its application; However, there are several other treatment strategies that can be used for manure treatment, such as chemical treatments,

liquid-solid separation, and biological processes (Fangueiro et al., 2021; Rodrigues et al., 2021).

Chemical treatment through pH modifications can be a suitable alternative. For instance, manure acidification, a common technique in Northern Europe for minimizing ammonia emissions, exhibits a significant impact on mitigating antibiotic resistance as well as reducing *E. coli* survival. Nonetheless, the associated corrosion and potential toxicity to plants hinders its global use. On the other end, alkaline treatments with products like quicklime (CaO) or hydrated lime ($Ca(OH)_2$) can effectively reduce pathogen concentration by disrupting pathogen cell membranes. However, resulting effluents would require neutralization prior to soil application (Fangueiro et al., 2021; Rodrigues et al., 2021).

Another common manure treatment is Solid-liquid separation, that promotes manure separation into a liquid and a solid fraction. The specific characteristics of the resulting fractions depend on the type of separation equipment used. Initially, the liquid manure undergoes a separation process, yielding two fractions: a liquid portion with around 3%–6% dry matter content, often used as fertilizer and a solid component. This separation process plays a crucial role in removing a substantial portion of tetracycline residues from the liquid fraction, as these antibiotics tend to bind to the solid fraction of the manure. Therefore, reducing the antibiotic load in the liquid fraction represents a critical initial step in manure treatment. In contrast, sulfonamides, owing to their limited absorption in the animal gut and/or reversible metabolization, are excreted in considerable quantities, persisting in the liquid manure, and subsequently entering the environment. The solid fraction can undergo further processing, including composting, drying, pelletization, or incineration. Meanwhile, the liquid fraction can be utilized as a fertilizer, although this use may contribute to the dispersion of antibiotic residues, ARG, and zoonotic bacteria into the environment. Both fractions have potential applications in anaerobic digestion (AD) systems for biogas production, which represents one of the most prevalent manure treatment methods in Europe (Fangueiro et al., 2021; Marutescu et al., 2022).

Biological processes constitute a vital aspect of manure treatment and encompass both aerobic and anaerobic techniques. Aerobic methods, exemplified by composting, play a pivotal role in stabilizing organic matter, mitigating odorous emissions, and eliminating pathogens present in the manure. Composting, as a specific form of aerobic biological treatment, has been documented to effectively reduce concentrations of antibiotics and antibiotic-resistant pathogens within manure. In contrast, AD is another biological process that not only produces biogas as an energy resource, but also lowers the levels of antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes in manure. Achieving effective pathogen inactivation during anaerobic digestion often requires the implementation of a thermophilic operational regime (Fangueiro et al., 2021; Marutescu et al., 2022). Studies

showed that postdigestion composting of manure reduces the presence of ARGs by more than 80%, suggesting that different manure management practices have different efficiencies in removing antibiotics, ARB, and ARG (Gurmessa et al., 2021; Marutescu et al., 2022).

Composting and AD are considered the most cost-effective means of processing manure before its spread onto agricultural soils, with both being associated with the reduction of pathogens and also of manure mass and volume, making its handling and transport easier (Jadeja & Worrich, 2022).

Despite the initial studies reporting that composting was more effective than anaerobic digestion in reducing antimicrobial residues, recent studies suggest that this removal is not complete. For instance, regarding cattle and dairy manure composting, a study showed that, while sulfamethazine and pirlimycin are almost fully removed by this treatment strategy, chlortetracycline removal can range between 71%–84%, tetracycline removal between 66%–72%, and tylosin removal is almost null (Jadeja & Worrich, 2022).

While conventional AD seems to be effective, especially in the removal of β-lactams, it shows poor results regarding sulfonamides, fluoroquinolones, and macrolides subtraction, being reported to have highly consistent removal rates near zero (Jadeja & Worrich, 2022; Marutescu et al., 2022). Overall, despite both methods being able to partially reduce some antibiotic residues, neither is efficient in eliminating them completely.

Similarly, to what is observed regarding antibiotics' elimination, AD and composting have mixed results regarding the removal of ARB and ARG. Studies point to the better efficiency of composting, especially when associated with higher temperatures. However, its efficiency highly depends on the type of manure and composting duration, with short-term composting of cattle manure having the worst results (Marutescu et al., 2022).

A promising solution for eliminating ARB and ARG from cattle manure could be its supplementation with biochar. Biochar results from the combustion of agricultural waste, municipal sludge, or other biomass under oxygen-limited conditions. This compound is especially advantageous when considering that agricultural wastes, such as rice husk, corn straw, and wheat straw, are widely distributed and easily available, producing biochar with a high carbon content (X. Zhang et al., 2023). Recently, the interest in biochar has spiked due to its multitude of uses, including the improvement of soil quality (Meyer et al., 2011), removal of emerging contaminants in water (Du et al., 2023; Son et al., 2018), and mitigation of greenhouse gas emissions (Creamer et al., 2014). Other advantages of this product include its low density, high stability, and strong adsorption capacity (Lyu et al., 2016). Furthermore, biochar can be modified by acids, bases, and oxidants, in order to improve the physicochemical properties of its surface (Ahmed et al., 2016), and it has been demonstrated to decrease the bioavailability of heavy metals and antibiotics via adsorption (Chen et al., 2018).

According to Jang and Kan (2022), the use of biochar can significantly remove ARGs (except for *tet*O and *erm*B) and genetic mobile elements such as *intI*1 from dairy manure (Jang & Kan, 2022). Furthermore, several studies suggest taking advantage of the porosity of biochar and combining it with other compounds to potentiate its ability to remove antibiotic residues and ARG (Du et al., 2023; Kang et al., 2022; Li et al., 2023). For instance, Kang *et al*. (2022) reviewed the efficacy of combining biochar with persulfate-based advanced oxidation process methods (persulfate-based-AOPs), concluding that biochar by itself was efficient in the removal of sulfamethoxazole, acetaminophen, and cephalexin, but its combination with persulfate based-AOPs provided more stable results (Kang et al., 2022).

A recent study by Li *et al* (2023) tested an association of biochar with peroxydisulfate for manure composting. This study showed highly promising results, with biochar potentiating the action of peroxydisulfate, resulting in a decrease of most ARG. Additionally, the resulting environment was not favorable to the proliferation of several microorganisms, such as those belonging to the genera *Thermopolyspora*, *Thermobifida*, and *Saccharomonospora*, contributing for decreasing the manure bacterial load and consequently ARG presence. The authors concluded that biochar-activated peroxydisulfate effectively reduced the risk of ARG transmission by optimizing the physicochemical characteristics of compost, reducing its moisture, adjusting the pH towards neutrality, and accelerating composting, which indirectly may lead to ARG decrease in manure (Li et al., 2023).

Another study by Jauregi *et al.* (2023) tested the amendment of soil with several compounds, including biochar, which was found to be one of the most promising compounds tested. In fact, the removal rate of *sul*1, *sul*2, *tet*A, and *intI*1 genes during composting using 5% of biochar was significantly higher than the one observed in the control group (Jauregi et al., 2023).

These studies support the application of biochar as a promising way of altering the composition of the bacterial community present in cow's manure, and consequently of the associated ARG and resistome profile.

2.1.7 Conclusions

In conclusion, AMR control in dairy farms has seen significant advancements in recent years. The adoption of a One-Health approach recognizes the interconnectedness of humans and food-producing animals in the spread of antibiotic-resistant bacteria and genes.

Three fundamental pillars for combating AMR have been elucidated:

1. **Monitoring and Control**: Global regulations, surveillance programs, and selective antimicrobial treatment strategies are vital to reduce unnecessary antibiotic use.
2. **Exploration of Alternative Antimicrobial Solutions**: Innovative alternatives like nisin and phage therapy offer promise in reducing reliance on traditional antibiotics, with nisin emerging as a particularly favorable option for dairy farms.

3. **Decontamination and Remediation Strategies**: Approaches like β-lactamases and molecular imprinting polymers (MIPs) are being explored to target specific antibiotics and remove residues. Biochar incorporation into manure shows the most potential in reducing AMR prevalence, however, it should be noted that it still needs further research to reach its full potential.

Efforts to combat AMR should be based on a global and multisectoral approach, including public education on antibiotic risks, stewardship programs for farmers and veterinarians, collaborations for antibiotic alternatives, and the development of remediation systems to eliminate AMR in dairy farm environments and milk.

In summary, addressing AMR comprehensively requires a multifaceted strategy that spans healthcare, agriculture, research, and environmental protection. By integrating these approaches, we can hope to slow the emergence and spread of antibiotic resistance, preserving these critical drugs for future generations.

2.1.8 Future Perspective:

From a forward-looking perspective for tackling AMR, the goals should focus on:

- **Enhanced Surveillance and Data Sharing:** Strengthening global surveillance programs for AMR across both human and animal populations is indispensable. Increased data sharing and collaboration between countries and organizations can provide a comprehensive understanding of resistance patterns and identify emerging threats.
- **Regulatory Measures and Stewardship Programs:** Governments and regulatory bodies must persist in implementing and enforcing stringent regulations governing antibiotic use. Concurrently, the promotion of stewardship programs among farmers and veterinarians can foster responsible antibiotic usage and monitoring.
- **Public Awareness and Education:** Expanding public education campaigns on the responsible use of antibiotics and the associated risks linked to AMR is of paramount importance. Raising awareness among consumers can significantly influence the demand for dairy products sourced from responsible antibiotic use.
- **Policy Alignment:** Encouraging alignment of policies and regulations across sectors and countries ensures a cohesive and coordinated approach to AMR mitigation, in both human and veterinary medicine.
- **Research Alternative Therapies:** Prioritize the development and validation of alternative antimicrobial therapies like peptides, nanoparticles, and phage therapy.
- **Precision Veterinary Medicine and Targeted Therapy:** Advances in molecular diagnostics and precision medicine present opportunities to tailor antibiotic treatment

regimens for individual animals. This personalized approach can minimize the indiscriminate use of broad-spectrum antibiotics, thereby reducing selective pressure for resistance.

- **Interdisciplinary Research:** Encouraging collaboration among researchers from diverse fields, including microbiology, veterinary medicine, agriculture, and environmental science, can lead to innovative, holistic solutions to address AMR.
- **Monitoring Antibiotic Residues:** Developing rapid and sensitive methods for monitoring antibiotic residues in dairy farms can help reduce the risk of human exposure to antibiotics through the food supply.
- **Global Partnerships:** Fostering partnerships between the pharmaceutical industry, research institutions, and governments is crucial to expedite the development and commercialization of viable antibiotic alternatives. Global collaboration is a driving force for innovation in this realm.
- **International Collaboration:** Fostering international collaboration is imperative to comprehensively address AMR on a global scale. Encouraging research partnerships between countries and regions is pivotal.
- **One-Health Approach:** Promoting the One-Health approach by supporting research and initiatives that recognize the interconnectedness of human, animal, and environmental health within the context of AMR is of major relevance.

In summary, addressing AMR in dairy farming and agriculture requires a multifaceted approach. Continued innovation and unwavering commitment from various stakeholders are indispensable to mitigate the looming threat of antimicrobial resistance.

2.1.9 Executive Summary:

Introduction

- Antibiotics were once a "magic bullet" against infectious diseases, improving life expectancy.
- Widespread use has led to AMR dissemination, creating a global crisis.
- Between 2000 and 2015, antibiotic consumption increased by 65%, reaching 34.8 billion tons.
- The emergence and dissemination of antibiotic resistance pose a significant threat, emphasizing the urgent need for further research to combat AMR in dairy farms.

The influence of antibiotic usage in farm animals on human health – a One-health Perspective.

- The One Health concept recognizes the interconnectedness of human, animal, and environmental health.
- Transmission of resistant bacteria, antibiotic residues, and resistance genes among

species is relevant.

- Collaborative efforts are required from human doctors, veterinarians, researchers, and the general population.

Antibiotic Usage in Cattle

- Globally, 93,309 tons of antimicrobials were administered to food-producing animals in 2017.
- The lowest quantity (42 mg/PCU) was used in cattle, with significant global variation.
- In the USA, 41% of antimicrobials sold for cattle are medically relevant.

Dairy Cattle Diseases and AMR:

- Antimicrobial use in dairy farms, in both milk-producing animals and young stock, should be carefully evaluated.
- **Mastitis:**
    - Inflammation of the mammary gland, common in dairy cattle, causes a major financial burden.
    - Pathogens responsible for mastitis include *S. aureus*, NAS, *Streptococcus* spp., and *E. coli*.
- **Reproductive Diseases:**
    - Metritis, especially puerperal metritis, is the second most common reason for antimicrobial treatment in dairy cows.
    - *Trueperella pyogenes* is a widespread opportunistic pathogen, associated with metritis and mastitis.
- **Hoof Diseases:**
    - Lameness impacts animal welfare and is the third most common cause of culling.
    - Digital dermatitis is associated with bacteria from the *Treponema genus*.
    - Bovine Foot Rot is caused by *Fusobacterium necrophorum*.
- **Bovine Respiratory Disease:**
    - *Mannheimia haemolytica, Histophilus somni, Pasteurella multocida, and Mycoplasma bovis* are major bacterial species.
    - Antimicrobials are the primary resort for controlling BRD.
- **Calf Diarrhea:**
    - Is a multifactorial disease with various pathogens.
    - *Salmonella enterica* and *E. coli* are major calf diarrhea pathogens associated with antimicrobial use.

AMR Spread Sources:

- Resistance spread is associated with biological excreta like milk, urine, and feces.

- **Waste Milk:**
    - Includes unmarketable milk, repurposed for calf feeding to reduce costs.
    - Can be a reservoir for ARB and ARG.
    - European Commission and EFSA recognize the risk of AMR spread through WM.
    - There are inconsistencies in research on the direct link between WM feeding and AMR development.
- **Manure:**
    - Its misuse or overuse can lead to environmental contamination with antibiotic residues, ARB, and ARG.
    - Antibiotic residues' effects depend on soil characteristics and climate.
    - Cattle manure is considered a reservoir for ARG with resistomes varying from herd to herd.

Strategies to Fight Antimicrobial Resistance

- **Monitoring and Control:**
    - Effective global regulations and surveillance programs are imperative to oversee and control antibiotic use.
    - Selective antimicrobial treatment, guided by culture testing, can reduce unnecessary antibiotic use, particularly in dairy farming contexts.
- **Innovative Alternatives:**
    - Nanoparticles, antimicrobial peptides like nisin, and phage therapy offer promising avenues to reduce reliance on antibiotics.
    - Nisin applications show considerable potential for practical implementation on dairy farms.
    - Further research is essential to validate their safety and effectiveness.
- **Decontamination and Remediation:**
    - **Milk:**
        - Strategies such as utilizing β-lactamases for targeted antibiotic degradation and MIPs for selective residue removal are promising.
    - **Manure:**
        - Implementing manure pretreatment methods, with a focus on biochar, holds substantial promise for the elimination of relevant bacteria.
        - Biochar's unique properties, including adsorption capacity, modification potential, selective ARG removal, and synergy with other treatments, make it a compelling solution to reduce the risk of soil contamination with ARB and ARGs.

3. Objectives

The growing need for sustainable manure management solutions in dairy farming, alongside the risks associated with AMR transmission and environmental contamination, drives the investigation of biochar supplementation in RMS.

Therefore, goal of this project was to evaluate the potential of biochar-supplemented RMS as a sustainable bedding material in dairy farming, with a focus on enhancing microbial safety, mitigating AMR transmission risks, and reducing environmental impact. To fulfill this goal, the study was organized around several key steps:

- Quantify *E. coli* and *Enterococcus spp*., as bacterial indicators, in biochar-supplemented RMS to gauge potential health risks, with strains and species an identification to understand possible microbial hazards.
- Evaluate the antibiotic resistance and virulence profiles of *E. coli* and *Enterococcus* isolates from biochar-supplemented RMS to assess the potential for antimicrobial resistance transmission and pathogenic potential.
- Assess microbial community shifts in biochar-supplemented RMS to determine its impact on microbial safety, focusing on overall changes in microbial populations of potential cow pathogens.
- Analyze greenhouse gas emissions and nutrient stability in biochar-supplemented RMS to evaluate its environmental safety and agronomic potential as a sustainable bedding material.

Chapter II

Potential of pine biochar to mitigate bacterial hazards present in recycled manure solids from dairy cows.

Ana José Pires, Ana Filipa Esteves, Gonçalo Pereira, Catarina Geraldes, Joana Fernandes Guerreiro[1,2], Raquel Abreu, Rita Magalhães, Lélia Chambel, Elisabete Silva, David Fangueiro, Manuela Oliveira, Ricardo Bexiga, 2025 Veterinary Sciences https://doi.org/10.3390/vetsci12010043

Authors' contributions:

Ana José Pires: performed the experiments, data analysis, original draft preparation, manuscript revision, and final approval.

Ana Filipa Esteves: contributed to the experimental work.

Catarina Geraldes: contributed to the experimental work and manuscript editing.

Lélia Chambel: contributed to methodology design, data curation, and manuscript editing.

Elisabete Silva: contributed to the methodology and manuscript revision.

Gonçalo Pereira: supported data analysis, formal statistics, and manuscript revision.

David Fangueiro: contributed to methodology validation, critical manuscript revision, supervision, and final approval.

Manuela Oliveira: responsible for project administration and funding acquisition; contributed to conceptualization, supervision, critical manuscript revision, and final approval.

Ricardo Bexiga: responsible for project administration and funding acquisition; contributed to conceptualization, supervision, critical manuscript revision, and final approval.

Simple Summary: The use of recycled manure solids as bedding for cattle can raise concerns about the spread of bacteria resistant to antibiotics, which could pose risks to both animal and human health. This study investigated whether adding biochar, a material produced by the pyrolysis of organic matter, to manure bedding can reduce harmful bacteria and minimize the risk of antibiotic resistance. The effect of supplementing manure solids with various biochar concentrations was tested in two time periods. The results indicate that the tested concentrations of biochar did not contribute to a reduction in RMS' bacterial loads nor in the antimicrobial resistance or virulence potential of the bacterial species analyzed. Further studies are necessary to evaluate biochar's ability to eliminate bacterial agents and determinants from manure solids before proceeding to its broader application in farming systems.

Abstract: The use of recycled manure solids (RMS) as cow bedding in dairy farms poses concerns due to its potential to harbor pathogenic and antimicrobial-resistant bacteria. This study evaluated the impact of RMS supplementation with biochar at three concentrations (2.5%, 5%, and 10%) on bacterial counts and on the antimicrobial resistance and virulence profiles of *Escherichia coli* and *Enterococcus* isolates. The results show that biochar supplementation did not promote a significant reduction in bacterial numbers. Overall, there were no significant changes in the isolates' virulence or resistance profiles, and none of the isolates obtained were classified as high or moderate threats based on their MAR and VIR indexes. The most pathogenic *Enterococcus* isolates found were present in the control samples. A phylogenetic analysis of *E. coli* isolates allowed us to identify phylogroup D strains, predominantly in RMS supplemented with 2.5% and 10% biochar, which are associated with higher virulence and resistance. These findings indicate that the biochar concentrations tested were not effective in significantly reducing the bacterial risks associated with RMS. Further research is needed to evaluate different biochar formulations and concentrations, aiming to optimize its potential use for RMS supplementation.

Keywords: dairy farms; recycled manure solids (RMS); biochar; antimicrobial resistance (AMR); virulence factors; *Escherichia coli*; *Enterococcus*

1. Introduction

In an era characterized by escalating global demands for animal-based food, the sustainability and safety of dairy farming practices have come under rigorous scrutiny. Of particular concern is the potential contribution of dairy farming to the dissemination of antimicrobial resistance (AMR), which poses significant environmental and health threats worldwide. The misuse of antibiotics in dairy farming creates selective pressure, driving the emergence of antimicrobial-

resistant bacteria (ARB). These resistant bacteria can spread through milk, manure, wastewater, and soil to the dairy farm environment, potentially affecting both animals and humans (Oliver et al., 2020; Pires et al., 2024). Furthermore, resistance genes can transfer across species and environments via horizontal gene transfer, complicating efforts to address the global challenge of AMR (Pires et al., 2024). Therefore, effective management practices are needed, particularly for cattle manure, which may serve as a reservoir for ARB and antimicrobial-resistant genes (ARG) due to intensive milk production practices that generate large volumes of manure rich in bacteria (Duan et al., 2019). These bacteria, whose counts usually range between 10^9 and 10^{10} colony-forming units per gram (CFU/g), may contribute to the environmental reservoir of resistance, underscoring the urgent need for effective mitigation strategies (Heinonen-Tanski et al., 2006; Leach et al., 2015).

Recycled manure solids (RMS) have gained popularity; they are used as bedding material for dairy cows due to their reduced costs and increased availability. However, the use of RMS raises concerns about bacterial transmission to the animals, including antimicrobial-resistant strains (Font-Palma, 2019). Unprocessed manure may harbor a diverse array of microbial populations, including potential pathogens, posing risks to animal health. While the practice of using manure as a soil amendment is prevalent worldwide, due to its nutrient-rich composition, high organic matter content, and cost-effectiveness for liquid manure disposal, its use in dairy farming warrants thorough investigation, which is imperative to assess the potential ramifications for the dissemination of AMR (Buta-Hubeny et al., 2022; Marutescu et al., 2022). To be used as cow bedding in Europe, RMS must adhere to Regulation 1069/2009 set forth by the European Parliament and the Council of the European Union. This regulation establishes a maximum threshold of 1000 *Enterobacteriaceae* CFU/g in manure by-products (Heinonen-Tanski et al., 2006). Several manure pre-treatment options, including chemical and physical treatments, can be applied to control bacterial levels in this product. However, these methods often fall short in completely eliminating antibiotic residues and pathogens. For instance, chemical treatments frequently fail to remove all antibiotic residues and may introduce additional chemicals into the environment (Beyers et al., 2022; Fangueiro et al., 2021; Font-Palma, 2019). On the other hand, solid–liquid separation is effective but expensive and energy-intensive (Fangueiro et al., 2012). Composting, despite reducing pathogen load, requires significant time and space and may not eliminate all antibiotic residues. Lastly, anaerobic digestion can leave behind resistant bacteria and genes due to suboptimal destruction conditions (Varma et al., 2021).

Bedding material can have serious implications in animals' health since dairy cows typically spend 40 to 65% of their time lying down, promoting direct contact between the mammary gland and the bacteria present in the bedding material, including *Streptococcus* spp. (e.g. *Streptococcus uberis*), coliform species (e.g. *Escherichia coli* and *Klebsiella* spp.),

Pseudomonas spp., and *Enterococcus* spp. (Klaas & Zadoks, 2018; Rowbotham & Ruegg, 2016), which can be responsible for intra-mammary infections and, therefore, bovine mastitis. Among the bacterial groups mentioned, the fecal indicator bacteria *E. coli* and enterococci are fundamental targets in studies exploring the use of RMS as cow bedding. Renowned for their robustness and prevalence in fecal matter, these organisms serve as indispensable proxies for assessing microbial contamination levels and associated health hazards inherent to the use of RMS as bedding material in bovine husbandry (Jacobs et al., 2019).

Besides being a relevant mastitis pathogen, *E. coli* is also associated with reproductive diseases and calf diarrhea in cattle and may pose a zoonotic risk. This potential is underscored by strains like Enterohemorrhagic *E. coli* (EHEC), which are of major public health concern. EHEC is linked to severe gastrointestinal diseases in humans, including hemorrhagic colitis and the potentially fatal hemolytic uremic syndrome (Kawasaki & Ambrosini, 2024). Cattle serve as the primary reservoir for EHEC, with human infections typically resulting from the consumption of contaminated meat and dairy products or from direct contact with infected animals. Moreover, while EHEC can cause life-threatening infections in humans, cattle, particularly adult cattle, often remain asymptomatic carriers, intermittently shedding the bacteria over extended periods (Kawasaki & Ambrosini, 2024; Kolenda et al., 2015).

Enterococci are another reliable indicator of fecal contamination, typically being commensal bacteria from the gastrointestinal tracts of animals and humans. They are also able to cause mastitis in cattle as well as nosocomial illnesses in humans, such as bacteremia, endocarditis, and urinary tract infections. Given their wide distribution and resilience in the environment, enterococci serve as reliable indicators of fecal contamination, playing a pivotal role in AMR surveillance systems for both human and animal health (Barlow et al., 2017; Ekore et al., 2022; Ji et al., 2023; Zaidi et al., 2022).

To reduce udder exposure to pathogenic bacteria and the incidence of associated infections, cost-effective methods are needed to control RMS microbiota (Fangueiro et al., 2017; Font-Palma, 2019; Heinonen-Tanski et al., 2006). Several studies have evaluated the efficacy of different compounds, including biochar, in reducing ARB in animals' manure. Biochar, a by-product of the pyrolysis of agricultural waste or other types of biomasses, has been described as a cost-effective product for use as an amendment of animal manure, including in dairy farms (Akdeniz, 2019; Chen et al., 2018; Cui et al., 2016; Du et al., 2023; Ma et al., 2024). Biochar represents a promising alternative due to its distinctive properties (Meyer et al., 2011). Its high surface area and porous structure enable it to adsorb a wide range of substances, including heavy metals, antibiotics, and organic pollutants, and it also has a noteworthy impact on microbial communities within manure (Akdeniz, 2019; Ma et al., 2024; Perez-Mercado et al., 2019). Specifically, biochar pores can serve as habitats for beneficial microorganisms, promoting their growth and activity while leading to the suppression of pathogenic organisms,

thereby improving soil health and reducing disease transmission risks (Akdeniz, 2019; Ma et al., 2024).

Before addressing its widespread use in dairy farms, RMS supplemented with biochar must be properly characterized concerning its microbial traits with the aim of evaluating its efficacy in inhibiting the dissemination of pathogenic and antimicrobial-resistant bacteria. As such, an incubation experiment with RMS supplemented with three different concentrations of biochar was performed over one month in two distinct time periods. To assess the impact of biochar supplementation on bacterial populations, RMS-supplemented samples from each condition (2.5%, 5%, and 10% biochar) were tested for the presence of *E. coli* and enterococci. Then, the antimicrobial susceptibility and virulence profiles of the isolated strains were analyzed to evaluate any changes attributable to the biochar treatments.

2. Materials and Methods

2.1 Incubation Experiment

An incubation experiment was conducted using fresh RMS from a commercial dairy farm in Portugal, obtained by mechanical separation. RMS was collected from the same commercial dairy farm at two time periods, namely April and June 2022 (Day 0). In the 30 days of the trials performed in each time period, the relative environmental humidity in the humid period (April–May) was much higher (71.5%) than in the dry period (June–July, 56.3%). The highest overall variations in both temperature and humidity were observed in the dry period (12.6 °C and 56.0% as opposed to 9.5 °C and 43.0% variations in the humid period) (https://www.ipma.pt/pt/index.html (accessed on 2 November 2024)). To avoid variability due to animal differences, only manure from one farm was used in both assays. The manure was collected directly after mechanical separation, mixed thoroughly to ensure homogeneity, and then divided into the appropriate experimental groups.

Subsequently, to test the influence of biochar supplementation on this product's microbiota, the obtained RMS was divided into five groups as follows: (1) non-supplemented RMS (negative control); (2) RMS supplemented with 10% H_2SO_4 (positive control, selected due to its known effectiveness in reducing bacterial counts through acidification); (3) RMS supplemented with 2.5% biochar (2.5B); (4) RMS with 5% biochar (5B); and (5) RMS with 10% biochar (10B) (percentages by weight (*w*/*w*)). Each group consisted of a total of 15 kg, representing three 5 kg replicates of RMS. Replicates from all groups were placed in identical and naturally ventilated containers and stored at ambient temperature for 30 days for each trial period: April–May 2022, corresponding to a more humid and cooler time period, and June–July 2022, a warmer and drier time period. The containers' contents were mixed every other day to ensure aeration.

The biochar used in this study was produced in Portugal, being obtained by pyrolysis of pine; however, the physicochemical characteristics (e.g., porosity, acidity, and particle size) were not disclosed by the biochar-producing company at the time of the study. The biochar used in all assays was from the same production batch.

2.2 Sample Collection

Ten grams of samples of RMS from all experimental groups was collected on days 0, 5, 15, and 30 of the two incubation trials. Sampling time points were selected to represent both the immediate and longer-term responses of the microbial populations to RMS supplementation. Day 0 represents the baseline, allowing for an initial assessment of the microbiota before any treatments had time to produce any effect. Day 5 was chosen to capture early microbial responses to biochar supplementations, as bacterial populations often begin to adjust within the first few days. Day 15 represents a midpoint, providing insights into the sustained effects of the treatments and any ongoing shifts in the microbial community. Finally, Day 30 was selected as the endpoint to assess the long-term impacts of the biochar supplementations, allowing for the evaluation of the stability of microbial changes over a one-month incubation period. These timepoints were intended to provide a comprehensive overview of bacterial growth and adaptation throughout the study.

Ten grams of biochar-supplemented samples, and those from the negative control, were suspended in 10 mL of saline solution and homogenized using a stomacher to obtain a 1:1 suspension that could be used for further processing. Subsequently, ten-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) in saline solution were carried out. After that, 100 µL of the suspensions were inoculated on the surface of MacConkey Agar (MAC) (VWR, Leuven, Belgium) and Slanetz and Bartley Agar (SB) (AppliChem, Darmstadt, Germany) plates using sterile glass beads. After 48 h of incubation at 37 °C, quantification of *Enterobacteriaceae* (total colonies on MAC) and of enterococci (total small round burgundy colonies on SB) was performed and expressed as CFU/g of bedding sample.

Then, up to four colonies presumptively identified as *E. coli* (lactose-fermenting colonies surrounded by a halo of bile salt precipitation on MAC) and as enterococci were collected from the plates corresponding to each experimental condition and replicate and inoculated onto Brain Heart Infusion (BHI) (VWR) agar plates, followed by incubation at 37 °C for 24 h.

All isolates were stored in buffered peptone water supplemented with glycerol (20%) (VWR) at −20 °C throughout the duration of the study.

2.3 Isolates' Identification and Molecular Characterization

For all molecular assays, a negative control (sterile PCR water) was included, and 10% of independent replicas were performed to validate and assess the results' reproducibility.

2.3.1 Enterococci

The phenotypical identification of the presumptive enterococci isolates was performed as previously described (Oliveira et al., 2016). Each isolate was inoculated on Bile Esculin (BE) agar (Scharlau, Barcelona, Spain) plates, a selective and differential medium for the isolation and identification of *Enterococcus* spp., and incubated at 37 °C for 42 h. Gram staining and catalase testing were performed for all the isolates that produced dark brown to black colonies in BE agar, aiming to detect Gram-positive and catalase-negative isolates, which were presumptively identified as *Enterococcus* spp. (Oliveira et al., 2016).

For molecular identification, the DNA from these isolates was obtained using the boiling method (Yamagishi et al., 2016). DNA purity and concentration were assessed using a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), followed by sample dilution to achieve a final DNA concentration of 50 ng/µL.

Genus identification followed an adaptation of the method described by Ke et al. (1999). The reaction mixtures, with a total volume of 25 µL, consisted of 12.5 µL of Supreme NZYTaq II 2x Green Master Mix (NZYTech, Lisbon, Portugal), 1 µM of each of the primers Ent1 and Ent2 (StabVida, FCT/UNL, Caparica, Portugal) (Table 1), and 1 µL of DNA [50 ng/µL] (Ke et al., 1999). Amplification was conducted using a XTender^{96} (VWR) thermocycler under the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 48 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. All PCR products were separated by agarose gel electrophoresis (1.3%, *w*/*v*) in 1x TBE buffer (NZYTech) supplemented with GreenSafe Premium (NZYTech). The electrophoresis process was conducted at 90 V for 1 h, and the outcomes were visualized using the ChemiDoc™ Gel Imaging System (Bio-Rad, San Diego, CA, USA).

Table 6 - Primers used for *Enterococcus* identification at genus and species levels and fingerprinting, and those used for *E. coli* fingerprinting and phylogroup identification.

Identification	Primer		Product Length	Reference
		Enterococcus		
Enterococcus spp.	Ent1 Ent 2	5' TACTGACAAACCATTCATGATG 3' 5' AACTTCGTCACCAACGCGAAC 3'	112 bp	(Ke et al., 1999)
E. faecium	FM1 FM2	5' GAAAAAACAATAGAAGAATTAT 3' 5' TGCTTTTTTGAATTCTTCTTTA 3'	215 bp	(Jackson et al., 2004)
E. faecalis	FL1 FL2	5' ACTTATGTGACTAACTTAACC 3' 5' TAATGGTGAATCTTGGTTTGG 3'	360 bp	
E. hirae	HI1 HI2	5' CTTTCTGATATGGATGCTGTC 3' 5' TAAATTCTTCCTTAAATGTTG 3'	187 bp	
E. durans	DU1 DU2	5' CCTACTGATATTAAGACAGCG 3' 5' TAATCCTAAGATAGGTGTTTG 3'	295 bp	
E. casseliflavus	CA1 CA2	5' TCCTGAATTAGGTGAAAAAAC 3' 5' GCTAGTTTACCGTCTTTAACG 3'	288 bp	
E. cecorum	CE1 CE2	5' AAACATCATAAAACCTATTTA 3' 5' AATGGTGAATCTTGGTTCGCA 3'	371 bp	
Fingerprinting	$(GTG)_5$	5' GTGGTGGTGGTGGTG 3'	200-3000 bp	(Semedo-Lemsaddek et al., 2013)
		E. coli		
Fingerprinting	ERIC2	5′ AAGTAAGTGACTGGGGTGAGCG 3′	380-3280 bp	(Silva et al., 2009)
gadA	Forward Reverse	5' GATGAAATGGCGTTGGCGCAAG 3' 5' GGCGGAAGTCCCAGACGATATCC 3'	373 bp	(Doumith et al., 2020)
chuA	Forward Reverse	5' ATGATCATCGCGGCGTGCTG 3' 5' AAACGCGCTCGCGCCTAAT 3'	281 bp	
yjaA	Forward Reverse	5' TGTTCGCGATCTTGAAAGCAAACGT 3' 5' ACCTGTGACAAACCGCCCTCA 3'	216 bp	
TSPE4.*C2*	Forward Reverse	5' GCGGGTGAGACAGAAACGCG 3' 5' TTGTCGTGAGTTGCGAACCCG 3'	152 bp	

Molecular identification of enterococci species was carried out using a multiplex PCR protocol adapted from (Jackson et al., 2004). The following species were targeted: *Enterococcus faecium* (primers FM1 and FM2), *Enterococcus faecalis* (FL1 and FL2), *Enterococcus hirae* (HI1 and HI2), *Enterococcus durans* (DU1 and DU2), *Enterococcus casseliflavus* (CA1 and CA2), and *Enterococcus cecorum* (CE1 and CE2) (Table 1). The following positive controls were used: *E. faecalis* ATCC 29212®, *E. faecium* CCUG 36804®, *E. hirae* ATCC 10541®, *E. durans* DSMZ 20633®, *E. cecorum* DSMZ 20682®, and *E. casseliflavus* DSMZ 20680®. A negative control (sterile PCR water) was included in each reaction. Additionally, 10% of independent replicates was tested for result validity and reproducibility.

For PCR reactions targeting *E. faecium* and *E. faecalis*, 25 µL reaction mixtures were prepared with 12.5 µL Supreme NZYTaq II 2x Green Master Mix (NZYTech), 1.25 µM of each primer, and 1 µL of DNA (50 ng/µL). Amplification was performed with the following cycle conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95 °C for 60 s, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. For the identification of *E. hirae*, *E. durans*, *E. cecorum*, and *E. casseliflavus*, PCR

mixtures containing 12.5 µL of Supreme NZYTaq II 2x Green Master Mix, 0.75 µM of each primer, and 2 µL of DNA (50 ng/µL) were used under similar amplification conditions, but we applied 30 cycles and an annealing temperature of 55 °C and final extension for 7 min (Jackson et al., 2004).

Genomic fingerprinting of enterococci was performed using a $(GTG)_5$ primer-based PCR method adapted from (Semedo-Lemsaddek et al., 2013). PCR reaction mixture contained 1x reaction buffer, 3 µM $MgCl_2$, 0.2 µM of each deoxynucleotide triphosphate, 2 µM of the primer, 0.06 U of Taq (Invitrogen, Waltham, MA, USA), and 100 ng of DNA. Amplification conditions included initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 60 s, annealing at 40 °C for 2 min, extension at 72 °C for 2 min, a final extension at 72 °C for 10 min (Semedo-Lemsaddek et al., 2013).

PCR products were separated by agarose gel electrophoresis (1.5%, *w*/*v*) in 0.5x TBE buffer, stained with GreenSafe Premium (NZYTech), and visualized using the ChemiDoc™ Gel Imaging System (Bio-Rad, Image Lab, Version 6.1.0). Genomic profiles were analyzed using hierarchical clustering with BioNumerics® 6.6 (Applied Maths, Kortrijk, Belgium).

2.3.2 E. coli

The presumptive identification of *E. coli* isolates was performed by Gram staining (Gram-negative non-sporulating bacillus), an oxidase test (oxidase-negative), and IMViC testing, which followed an adapted protocol derived from Fernandes et al. (2022) focusing on testing for indole, motility, Voges–Proskauer, and citrate utilization (Fernandes et al., 2022). Isolates that exhibited a positive reaction for indole and motility, along with a negative reaction for Voges–Proskauer and citrate, were presumptively identified as *E. coli* and subjected to genomic fingerprinting and phylogenetic grouping.

DNA from the *E. coli* isolates was obtained utilizing the boiling method (Yamagishi et al., 2016). DNA purity and concentration were assessed using a Nanodrop spectrophotometer (ThermoFisher Scientific), and all samples were diluted to achieve a final DNA concentration of 50 ng/µL. Isolates' genomic fingerprinting was accomplished through ERIC-PCR following the methodology outlined by Silva et al. (2009). The PCR mixture consisted of 10 µL of sterile PCR water, 12.5 µL of MasterMix (NZYTech), 0.5 µL of the ERIC2 primer (Table 1) (Stabvida), and 2 µL of template DNA [50 ng/µL] for a final volume of 25 µL. Amplification was conducted using an XTender^{96} (VWR) thermocycler under the following conditions: initial denaturation at 95 °C for 7 min, followed by 30 cycles of denaturation at 90 °C for 30 s, annealing at 52 °C for 60 s, extension at 72 °C for 8 min, and a final extension at 72 °C for 16 min (Silva et al., 2009). All products were separated by agarose gel electrophoresis (1.5%, *w*/*v*) in 0.5x TBE buffer (NZYTech) supplemented with GreenSafe Premium (NZYTech). Electrophoresis was conducted at 70 V for 2 h.

As for enterococci, all electrophoresis outcomes were visualized using the ChemiDoc™ Gel Imaging System (Bio-Rad), and fingerprinting results were assessed using BioNumerics® 6.6 (Applied Maths) as previously described.

Phylogenetic grouping of the *E. coli* isolates was performed using primers focusing on the *gadA*, *chuA*, and *yjaA* genes and the DNA fragment TSPE4-*C2* (Stabvida) (Table 1) (Doumith et al., 2020). PCR was conducted in a reaction volume of 20 µL, comprising 0.4 µL of each primer with a final concentration of 1 µM, 10 µL of Master Mix (NZYTech), 5.8 µL of sterile PCR water, and 1 µL of bacterial DNA [200 ng/µL]. Amplification was conducted using an XTender^{96} (VWR) thermocycler under the following conditions: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min (Doumith et al., 2020). Three positive controls (*E. coli* strain J96 belonging to phylogenetic group B2, *E. coli* strain KS52 belonging to phylogenetic group A, and *E. coli* strain 22.8 D belonging to phylogenetic group D) were included.

PCR products underwent separation through agarose gel electrophoresis (2%, *w*/*v*) in 0.5x TBE buffer (NZYTech) supplemented with GreenSafe Premium (NZYTech). Electrophoresis was carried out at 70 V for 2 h, and the results were observed as previously described.

2.4 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of the *Enterococcus* spp. and *E. coli* representative isolates selected based on PCR fingerprinting was performed using the disk diffusion method following the Clinical and Laboratory Standards Institute (CLSI) standards (Clinical and Laboratory Standards Institute., 2024, 2024). To ensure the validity and reproducibility of the assays, 10% independent replicas were performed. Reference strains *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *E. faecalis* ATCC 29212 were used as control strains.

The antibiotics tested were selected based on their frequent use on dairy farms and in human medicine and included compounds from several antibiotic classes. For *Enterococcus* spp., the antibiotics tested included penicillins (ampicillin, 10 µg; amoxicillin–clavulanic acid, 20/10 µg); glycopeptides (vancomycin, 30 µg); tetracyclines (oxytetracycline, 30 µg); aminoglycosides (high-dose gentamicin, 120 µg); and fluoroquinolones (enrofloxacin, 5 µg) (Oxoid Limited®, Hampshire, UK). For *E. coli*, the antibiotics tested included penicillins (ampicillin, 10 µg; amoxicillin–clavulanic acid 20/10 µg); tetracyclines (oxytetracycline, 30 µg); sulphonamides (trimethoprim/sulfamethoxazole, 1.25/23.75 µg); fluoroquinolones (enrofloxacin, 5 µg); and cephalosporins (ceftiofur, 30 µg) (Oxoid Limited®).

Bacterial suspensions, with a turbidity equivalent to 0.5 on the McFarland scale (equivalent to approximately 1.5×10^8 CFU/mL), were inoculated using the lawn technique on the surface of Mueller–Hinton agar (Oxoid Limited®) plates. Antibiotic disks were then placed on the agar

surface, and plates were incubated for 18 h at 36 °C, except for vancomycin testing plates, which were incubated for 24 h at the same temperature. After incubation, the diameters of the inhibition halo around the disks were measured, and results were interpreted according to CLSI guidelines M31 A3 (Clinical and Laboratory Standards Institute., 2008), VET09 (Clinical and Laboratory Standards Institute., 2024), and M100 (Clinical and Laboratory Standards Institute., 2024).

The Multiple Antibiotic Resistance (MAR) index for each isolate was calculated according to Singh et al. (2017), considering the number of antimicrobials to which the isolates were resistant to divided by the number of antimicrobials tested. An average of each MAR was then calculated for the isolates from each treatment condition (Singh et al., 2017).

2.5 Virulence Assays

The phenotypic virulence profile of all representative isolates was established by assessing their ability to produce hemolysin, gelatinase, biofilm, DNase, proteinase, and lecithinase using specific media following the procedures used by Fernandes et al. (2022) (Fernandes et al., 2022). Positive and negative controls, along with 10% independent replicas, were also tested.

Hemolysin production was evaluated on Columbia agar medium supplemented with 5% sheep blood (bioMérieux, Marcy-l'étoile, France) using *S. aureus* ATCC® 25923 as positive control and *E. coli* ATCC® 25922 as negative control. A positive reaction was indicated by the formation of a halo around the colonies after incubation at 37 °C for 72 h.

Gelatinase activity was assessed using nutrient gelatin agar (Oxoid Limited ®), with *Pseudomonas aeruginosa* ATCC® 27853 as positive control and *E. coli* ATCC® 25922 as negative control. After an incubation period of 48 h at 37 °C, the cultures were refrigerated for 30 min at 4 °C, with liquefaction of the medium being considered a positive result.

Biofilm formation was evaluated in BHI agar medium (VWR) supplemented with 0.8% Congo Red (Sigma-Aldrich, St. Louis, MO, USA) and 5% sucrose (Millipore Sigma-Aldrich, ON, Canada), with *E. faecium* ATCC® 29212 being used as positive control and *E. coli* ATCC® 25922 as negative control. Positive reactions were characterized by the formation of colonies ranging from brown to black after incubation at 37 °C for 72 h.

DNase activity was assessed in DNase agar (VWR) supplemented with 0.01% toluidine blue (Merck KGaA, Darmstadt, Germany), using *S. aureus* ATCC® 25923 as positive control and *E. coli* ATCC® 25922 as negative control. A pink halo around the colonies after incubation at 37 °C for 72 h indicated a positive reaction.

Proteinase activity was evaluated in Skim Milk Agar (VWR), testing *P. aeruginosa* ATCC® 27853 as positive control and *S. aureus* ATCC® 29213 as negative control. The presence of

a transparent halo around the colonies after 48 h of incubation at 37 °C denoted a positive reaction.

Lecithinase activity was assessed in Tryptic Soy Agar (VWR) supplemented with 10% egg yolk emulsion (VWR), with *P. aeruginosa* ATCC® 27853 being used as positive control and *E. coli* ATCC® 25922 as negative control. A positive reaction resulted in the formation of a white precipitation halo around the colonies after incubation at 37 °C for 72 h.

The virulence index (VIR) for each isolate was determined by dividing the sum of all positive virulence phenotypes exhibited by the isolates by the total number of virulence factors tested (Singh et al., 2017). An average of each VIR was then calculated for each treatment.

Finally, isolates were classified according to their threat level. According to the MAR and VIR classification system created by Singh et al. (2017) (Singh et al., 2017), isolates can be categorized as a high threat if they exhibit a MAR index ≥ 0.30 and a VIR index ≥ 0.50; as a moderate threat if the MAR index is < 0.30 and the VIR index is ≥ 0.50; as a low threat if the MAR index is ≥ 0.30 and the VIR index is < 0.50; and, finally, as no threat if the MAR is < 0.30 and the VIR index is < 0.50.

2.6 Data Analysis

To evaluate the effect of biochar supplementation on bacterial counts, specifically of *Enterococcus* spp. and *Enterobacteriaceae*, a comprehensive statistical analysis was performed using SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) (Zdanowicz et al., 2004). The bacterial counts were first adjusted by adding a constant of 1, and then they were log-transformed to normalize the data. A linear mixed-effects model (PROC MIX, SAS) was employed to account for the repeated measures design of the study. The model included treatment (C−, C+, 2.5%, 5%, and 10%) and time (0, 5, 15, and 30) as fixed effects, with replicate as a random effect. This model allowed us to account for the hierarchical structure of the data and the correlation between repeated measurements on the same replicate. The Kenward–Roger method was used for degrees of freedom calculation to improve the accuracy of the fixed-effect tests. Least squares means (LS-means) were computed for each treatment and time point, and pairwise comparisons were adjusted using the Tukey method to control the family-wise error rate. The covariance structures used were the ones resulting in the lowest Akaike information criteria, CS (compound symmetry) for *Enterococcus* and AR (1) (first-order autoregressive) for *Enterobacteriaceae*. The significance of biochar treatments in the isolates' antibiotic susceptibility and virulence profiles was determined using a generalized linear mixed model with a logit link and binary distribution for the outcome variable, following the PROC GLIMMIX procedure in SAS. Manual backward elimination, guided by a *p*-value threshold of 0.157 as suggested by Heinze and Dunkler (2017), was conducted without initially filtering individual variables to establish the final models (Heinze & Dunkler, 2017). The variable "condition" was included in the model regardless of its statistical significance. In the final

models, differences were considered significant when $p \leq 0.05$. The distribution of the different *E. coli* phylogenetics groups was analyzed with Chi-Square Test of Independence (PROC FREQ).

Given that the MAR and virulence indexes could only assume one of the following values (0.10, 0.11, 0.12, 0.18, 0.19, 0.23, and 0.33 for the MAR index and 0.17, 0.19, 0.22, 0.23, 0.26, 0.32, and 0.37 for the virulence index), they were analyzed as ordinal response variables and fitted to cumulative logistic regression models (PROC LOGISTIC).

3. Results

3.1 Bacteria Quantification

The raw data regarding bacterial counts in both CFU/g and log units are available in Tables S2 and S3 in the Supplementary Files.

Results from bacterial quantification on the RMS samples supplemented with biochar (2.5%, 5%, and 10%) did not show significant differences from those from the control group. The bar charts in Figure 1 illustrate the mean *Enterobacteriaceae* counts across different treatments and time points. As depicted, *Enterobacteriaceae* counts showed different trends. In the assay performed during the dry period (June–July), a log decrease was observed by Day 30 in the negative control (of 0.28 log units), as well as in the RMS supplemented with 5% biochar (of 0.13 log units) and in the RMS supplemented with 10% biochar (of 0.27 log units); on the contrary, a log increase was observed by Day 30 in the RMS samples supplemented with 2.5% biochar (of 0.58 log increase). The same tendency was observed in the assay performed during the wet period (April–May) by Day 30, in which all RMS samples (including the negative controls and the biochar-supplemented samples) showed an increase in *Enterobacteriaceae* log units, ranging from 0.43 (for the samples supplemented with 10% biochar) to 1.91 (for the samples supplemented with 2.5% biochar).

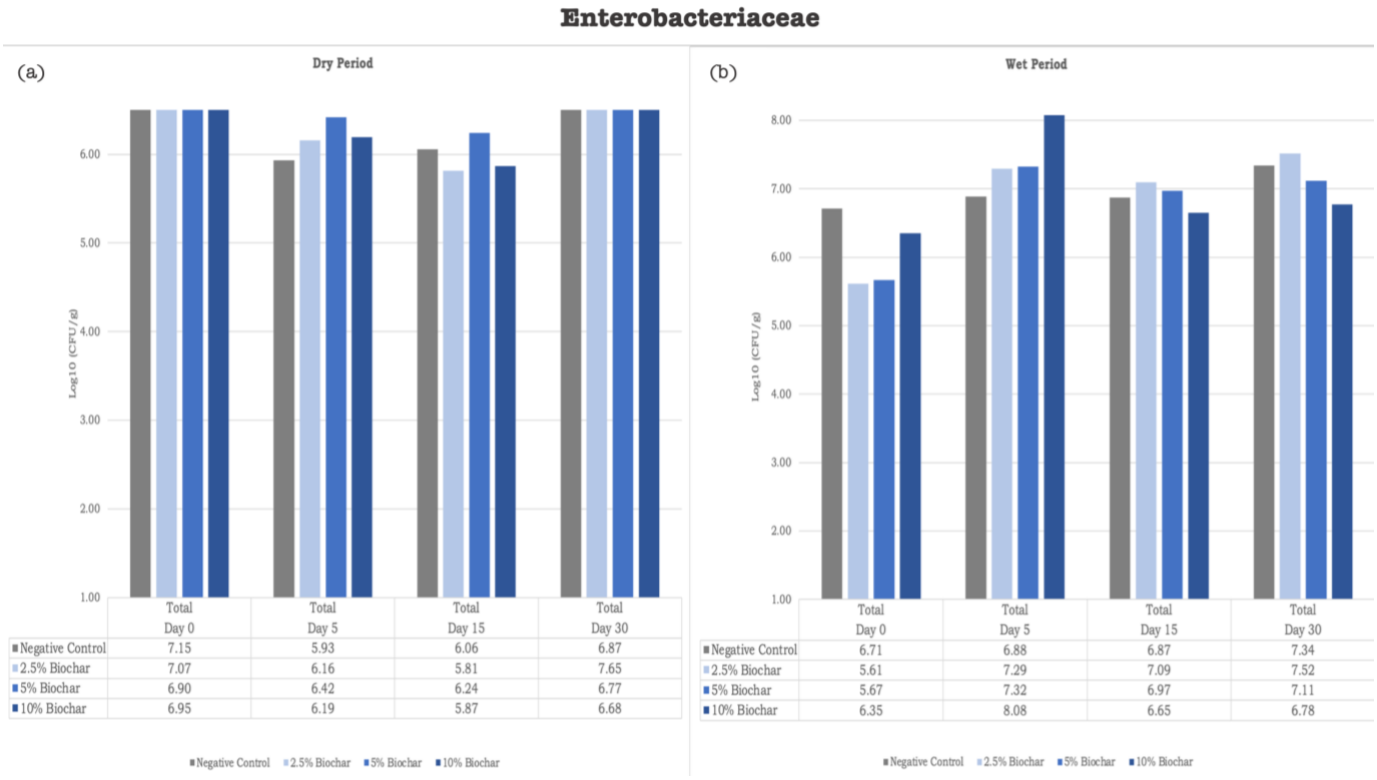
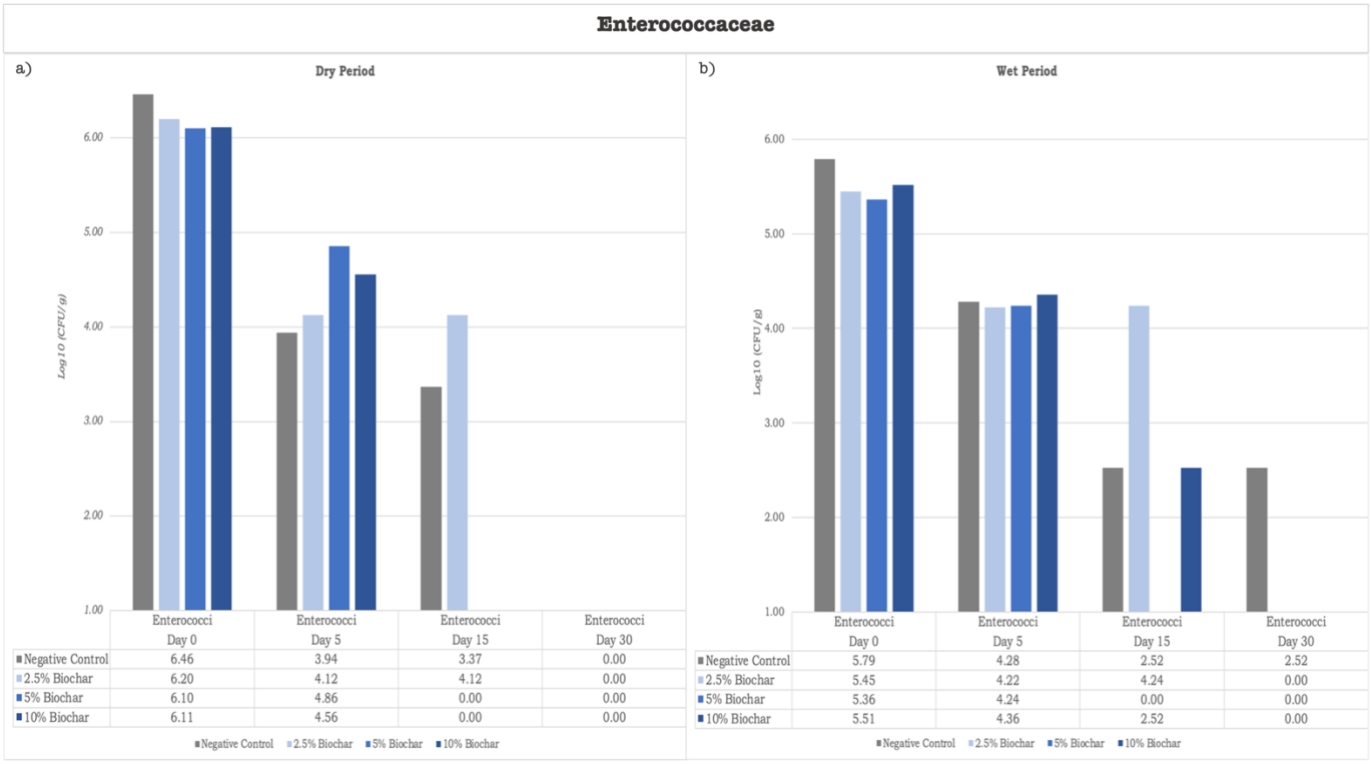


Figure 1 . Graphic representation of *Enterobacteriaceae* quantification [Log10(CFU/g)]. (**a**) Bacteria quantification [Log10(CFU/g)] in MAC inoculated with samples collected during assay performed in dry period; (**b**) bacteria quantification [Log10(CFU/g)] in MAC inoculated with samples collected during assay performed in wet period. Logarithmic values were calculated based on average CFU obtained from three replicates for each treatment and time point.

Figure 2 illustrates the mean *Enterococcaceae* counts across different treatments and time points. By Day 30, a decrease in *Enterococcaceae* log units was observed in the two assays for all the conditions tested. In the assay performed during the dry period (June–July), the log decreases observed ranged between 6.10 log in the RMS samples supplemented with 5% biochar and 6.46 log in the negative control samples, while in the assay performed during the wet period (April–May), the log decreases observed ranged between 3.27 log in the negative control samples and 5.51 log in the RMS samples supplemented with 10% biochar.



	Enterococci Day 0	Enterococci Day 5	Enterococci Day 15	Enterococci Day 30
Negative Control	6.46	3.94	3.37	0.00
2.5% Biochar	6.20	4.12	4.12	0.00
5% Biochar	6.10	4.86	0.00	0.00
10% Biochar	6.11	4.56	0.00	0.00

	Enterococci Day 0	Enterococci Day 5	Enterococci Day 15	Enterococci Day 30
Negative Control	5.79	4.28	2.52	2.52
2.5% Biochar	5.45	4.22	4.24	0.00
5% Biochar	5.36	4.24	0.00	0.00
10% Biochar	5.51	4.36	2.52	0.00

Figure 2 - Graphic representation of *Enterococcaceae* quantification [Log10(CFU/g)] a) bacteria quantification [Log10(CFU/g)] in SB inoculated with samples collected during the assay performed in the wet period b) bacteria quantification [Log10(CFU/g)] in SB inoculated with samples collected during the assay performed in the dry period. Logarithmic values were calculated based on the average CFU obtained from three replicates for each treatment and time point.

As seen in Table 7, significant reductions in *Enterococcus* spp. counts were observed over time. However, the effects of biochar supplementation at 2.5%, 5%, and 10% on reducing *Enterococcus* spp. counts were not statistically significant. Similarly, for *Enterobacteriaceae*, no significant differences were found between biochar treatments and the negative control for either bacterial group.

Table 7 - Summary of Comparisons for *Enterococcus* spp. and *Enterobacteriaceae* Counts - This table presents the post-hoc comparisons of log-transformed bacterial counts for both *Enterococcus* spp. and *Enterobacteriaceae* across different treatments, time points. The estimates indicate the differences in log counts between the specified groups, adjusted p-values. Significant comparisons are highlighted in green, demonstrating the effects of time and treatment on bacterial reduction.

Comparisons	***Enterococcus* spp. p-value**	***Enterobacteriaceae* p-value**
Day 0 vs. Day 5	<0.0001	0.9715
Day 0 vs. Day 15	<0.0001	0.9436
Day 0 vs. Day 30	<0.0001	0.4090
Negative Control vs. 2.5% Biochar	0.7346	0.8808
Negative Control vs. 5% Biochar	0.1156	0.9951
Negative Control vs. 10% Biochar	0.6659	0.9967

3.2 *Enterococcus* identification and molecular characterization

From the incubation assay, a total of 103 presumptive enterococci isolates were obtained from all RMS treatment groups during the assay performed in the wet period, from which only 31 were confirmed as belonging to the genus *Enterococcus* by phenotypic tests. Similarly, during the assay performed in the dry period, 109 presumptive enterococci isolates were collected from all RMS treatment groups, with 22 being phenotypically confirmed as belonging to the genus *Enterococcus*. Of these 53 isolates, 50 were confirmed as belonging to the genus *Enterococcus* by PCR and further identified to the species level.

In this study, the identification of *Enterococcus* isolates at species level, including *E. faecium, E. faecalis*, *E. hirae*, and *E. durans*, was achieved using specific primers designed for each target species. However, for *E. cecorum* and *E. casseliflavus,* the size of the PCR products obtained did not match the expected sizes for either species (371 bp and 288 bp, respectively). Subsequently, the PCR products were subjected to a sequencing analysis, and the isolates' identification as *E. gallinarum* was confirmed using the BLAST (Basic Local Alignment Search Tool) algorithm, as seen in Table S1 in the Supplementary Files.

As observed in Table 8, *E. faecium* was detected only in the wet period, with 16% being found in the negative control samples, 3% in the samples of RMS supplemented with 5% biochar, and 6% in the samples of RMS supplemented with 10% biochar. *E. faecalis* appeared in 6% of the samples of RMS supplemented with 5% biochar during the wet period. *E. hirae* showed a 32% prevalence in the samples of RMS supplemented with 2.5% biochar during the dry period and also appeared in 11% of the samples with 5% biochar. *E. gallinarum* was identified in 13% of the samples of RMS supplemented with 2.5% biochar and 16% in the samples of RMS supplemented with 10% biochar during the wet period, whereas it showed a 16% prevalence in the negative control during the dry period. *Enterococcus* sp. had a higher

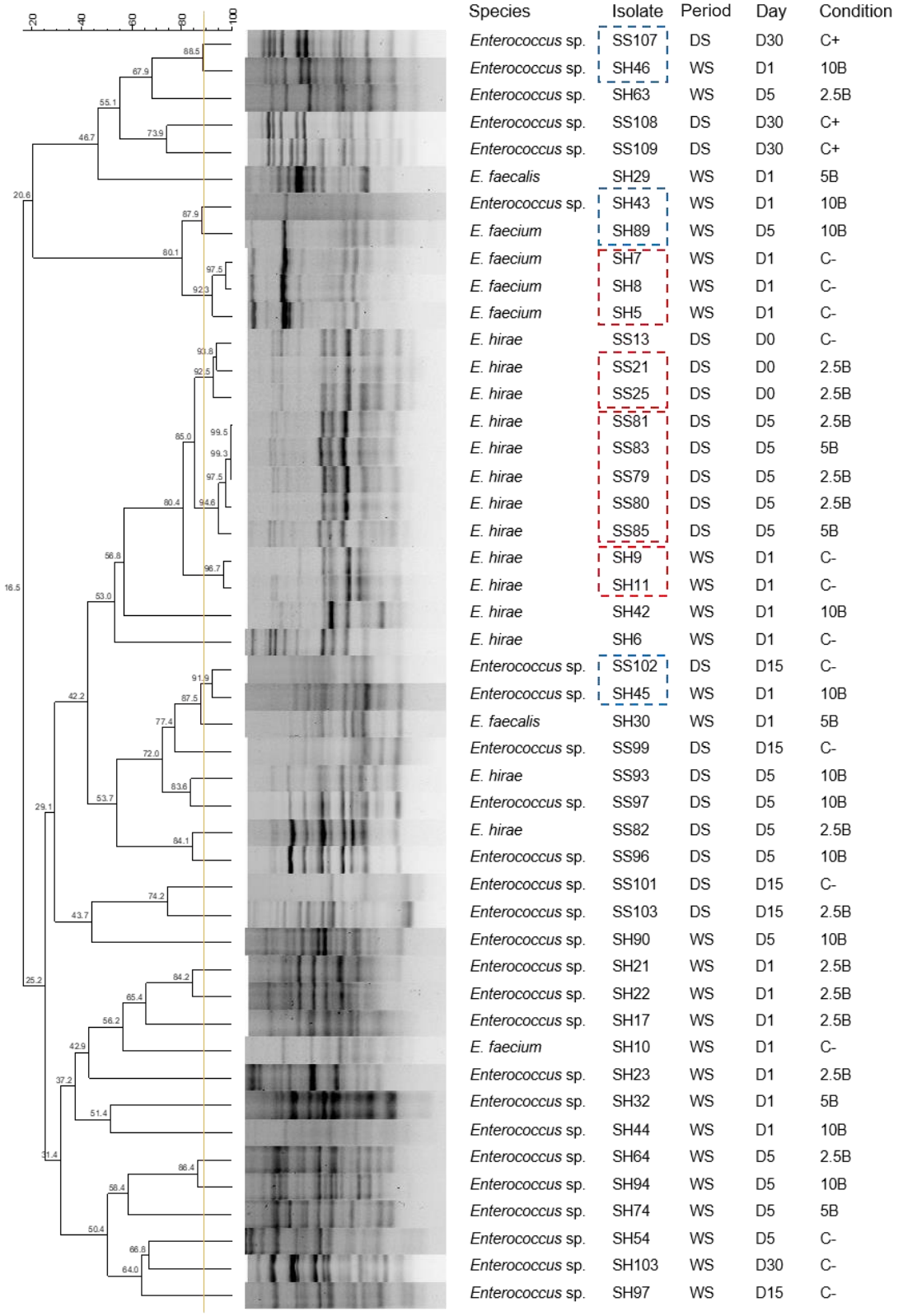
presence in the wet period, with 10% being found in the negative control samples and 6% in the samples of RMS supplemented with 2.5% and 5% biochar. In the dry period, it appeared in 11% of the negative control and 11% of the samples with 10% biochar. The samples with fewer occurrences of the *Enterococcus* species known for their elevated health risks, *E. faecium* and *E. faecalis*, were the samples of RMS supplemented with 2.5% biochar during the dry period.

Table 8 - The species distribution (%) of *Enterococcus* in RMS for each condition. The overall percentage represents the sum of isolates for both periods divided by the total number of isolates for both periods. C+—positive control; C−—negative control; 2.5B—RSM supplemented with 2.5% biochar; 5B—RSM supplemented with 5% biochar; 10B—RSM supplemented with 10% biochar.

	Period	C+	C-	2.5B	5B	10B
E. faecium	Wet	0	16	0	3	6
	Dry	0	0	0	0	0
	Overall	0	10	0	2	4
E. faecalis	Wet	0	0	0	6	0
	Dry	0	0	0	0	0
	Overall	0	0	0	4	0
E. durans	Wet	0	0	0	0	0
	Dry	0	0	0	0	0
	Overall	0	0	0	0	0
E. hirae	Wet	0	10	0	0	3
	Dry	0	5	32	11	5
	Overall	0	6	12	4	4
E. gallinarum	Wet	0	0	13	0	16
	Dry	16	5	5	0	0
	Overall	6	0	10	0	10
Enterococcus sp.	Wet	0	10	6	6	3
	Dry	0	11	0	0	11
	Overall	0	8	6	4	6

The $(GTG)_5$ fingerprinting analysis allowed to compare the profiles of *Enterococcus* isolates and select 40 representative isolates for further tasks. Using a reproducibility cut-off of 88.5%, the isolates with identical fingerprinting profiles, obtained from the samples collected in the same period, treatment group, and sampling day, were excluded.

The dendrogram (Figure 3) revealed clonal relationships among the *Enterococcus* isolates from the RMS samples supplemented with biochar under different conditions. Isolates from the wet period (WS) and dry period (DS) were analyzed for clonal similarity. Globally, there are isolates with different profiles indicating a level of genetic diversity, with seven clusters representing clonal relationships (≥ 85% similarity).



Species	Isolate	Period	Day	Condition
Enterococcus sp.	SS107	DS	D30	C+
Enterococcus sp.	SH46	WS	D1	10B
Enterococcus sp.	SH63	WS	D5	2.5B
Enterococcus sp.	SS108	DS	D30	C+
Enterococcus sp.	SS109	DS	D30	C+
E. faecalis	SH29	WS	D1	5B
Enterococcus sp.	SH43	WS	D1	10B
E. faecium	SH89	WS	D5	10B
E. faecium	SH7	WS	D1	C-
E. faecium	SH8	WS	D1	C-
E. faecium	SH5	WS	D1	C-
E. hirae	SS13	DS	D0	C-
E. hirae	SS21	DS	D0	2.5B
E. hirae	SS25	DS	D0	2.5B
E. hirae	SS81	DS	D5	2.5B
E. hirae	SS83	DS	D5	5B
E. hirae	SS79	DS	D5	2.5B
E. hirae	SS80	DS	D5	2.5B
E. hirae	SS85	DS	D5	5B
E. hirae	SH9	WS	D1	C-
E. hirae	SH11	WS	D1	C-
E. hirae	SH42	WS	D1	10B
E. hirae	SH6	WS	D1	C-
Enterococcus sp.	SS102	DS	D15	C-
Enterococcus sp.	SH45	WS	D1	10B
E. faecalis	SH30	WS	D1	5B
Enterococcus sp.	SS99	DS	D15	C-
E. hirae	SS93	DS	D5	10B
Enterococcus sp.	SS97	DS	D5	10B
E. hirae	SS82	DS	D5	2.5B
Enterococcus sp.	SS96	DS	D5	10B
Enterococcus sp.	SS101	DS	D15	C-
Enterococcus sp.	SS103	DS	D15	2.5B
Enterococcus sp.	SH90	WS	D5	10B
Enterococcus sp.	SH21	WS	D1	2.5B
Enterococcus sp.	SH22	WS	D1	2.5B
Enterococcus sp.	SH17	WS	D1	2.5B
E. faecium	SH10	WS	D1	C-
Enterococcus sp.	SH23	WS	D1	2.5B
Enterococcus sp.	SH32	WS	D1	5B
Enterococcus sp.	SH44	WS	D1	10B
Enterococcus sp.	SH64	WS	D5	2.5B
Enterococcus sp.	SH94	WS	D5	10B
Enterococcus sp.	SH74	WS	D5	5B
Enterococcus sp.	SH54	WS	D5	C-
Enterococcus sp.	SH103	WS	D30	C-
Enterococcus sp.	SH97	WS	D15	C-

Figure 3 - Dendrogram of *Enterococcus* isolates obtained using $(GTG)_5$ molecular fingerprinting. Isolates within red dashed rectangles (SH5, SH7, SH9, SS21, SS79, SS80, and SS83) were excluded due to their clonal similarity and matching collection parameters. However, isolates within blue dashed rectangles, despite being clones, were not excluded as they were collected in different periods, days, or conditions. Yellow line—cut-off line of 88.5% (reproducibility value). D—day of sample collection; C+—positive control; C−—negative control; 2.5B—RMS supplemented with 2.5% biochar; 5B—RMS supplemented with 5% biochar; 10B—RMS supplemented with 10% biochar; WS—wet period DS—dry period.

3.3 *E. coli* identification and molecular characterization

The sample culture yielded 192 and 170 presumptive *E. coli* isolates from assays performed during the wet and dry periods, respectively. Using the phenotypic identification method described in the Materials and Methods Section, 28 and 44 isolates were confirmed as *E. coli* from the wet and dry periods, respectively. This discrepancy between presumptive and confirmed isolates highlights the challenges of accurate identification. The method involved an initial selection based on colony morphology on MacConkey agar, followed by biochemical tests. However, false positives can occur due to non-target bacteria exhibiting similar phenotypic traits (Yong Ng et al., 2010).

Using ERIC fingerprinting, it was possible to compare the profiles of the 72 *E. coli* isolates and select 42 representatives for further analysis based on a reproducibility cut-off of 68.8%.

The dendrogram (Figure 4) illustrates the relationships among *E. coli* isolates from RMS samples. Some genetic diversity is observed, but there are also major clusters with ≥68.8% similarity, containing isolates from different periods and biochar supplementation.

The results from the fingerprinting assay allowed us to exclude isolates with the same fingerprinting profile that were obtained from samples collected in the same period, treatment group, and sampling day.

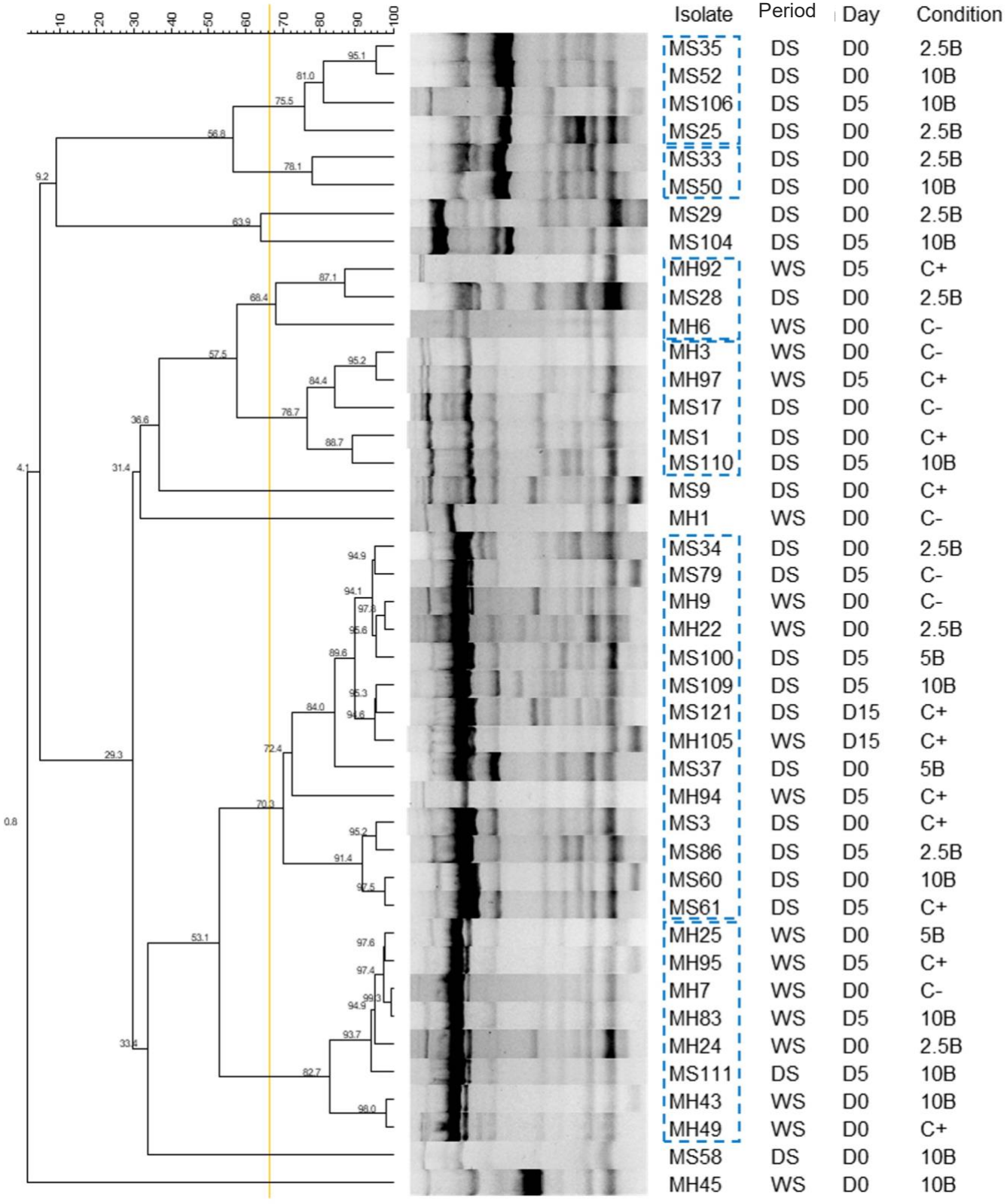


Figure 4 - Dendrogram of representative *E. coli* isolates obtained using REP–PCR molecular fingerprinting. Isolates within blue dashed rectangles, despite being clones, were not excluded as they were collected in different periods, days, or conditions.
Yellow line – Cut-off line of 68.8%. D – Day of sample collection; C+ – Positive Control; C- – Negative Control; 2.5B – RSM supplemented with 2.5% biochar; 5B – RSM supplemented with 5% biochar; 10B – RSM supplemented with 10% biochar; WS -Wet Period DS – Dry Period.

The phylogenetic prevalence of *E. coli* isolates in the RMS samples, depicted in Figure 5, shows distinct trends across different conditions and periods.

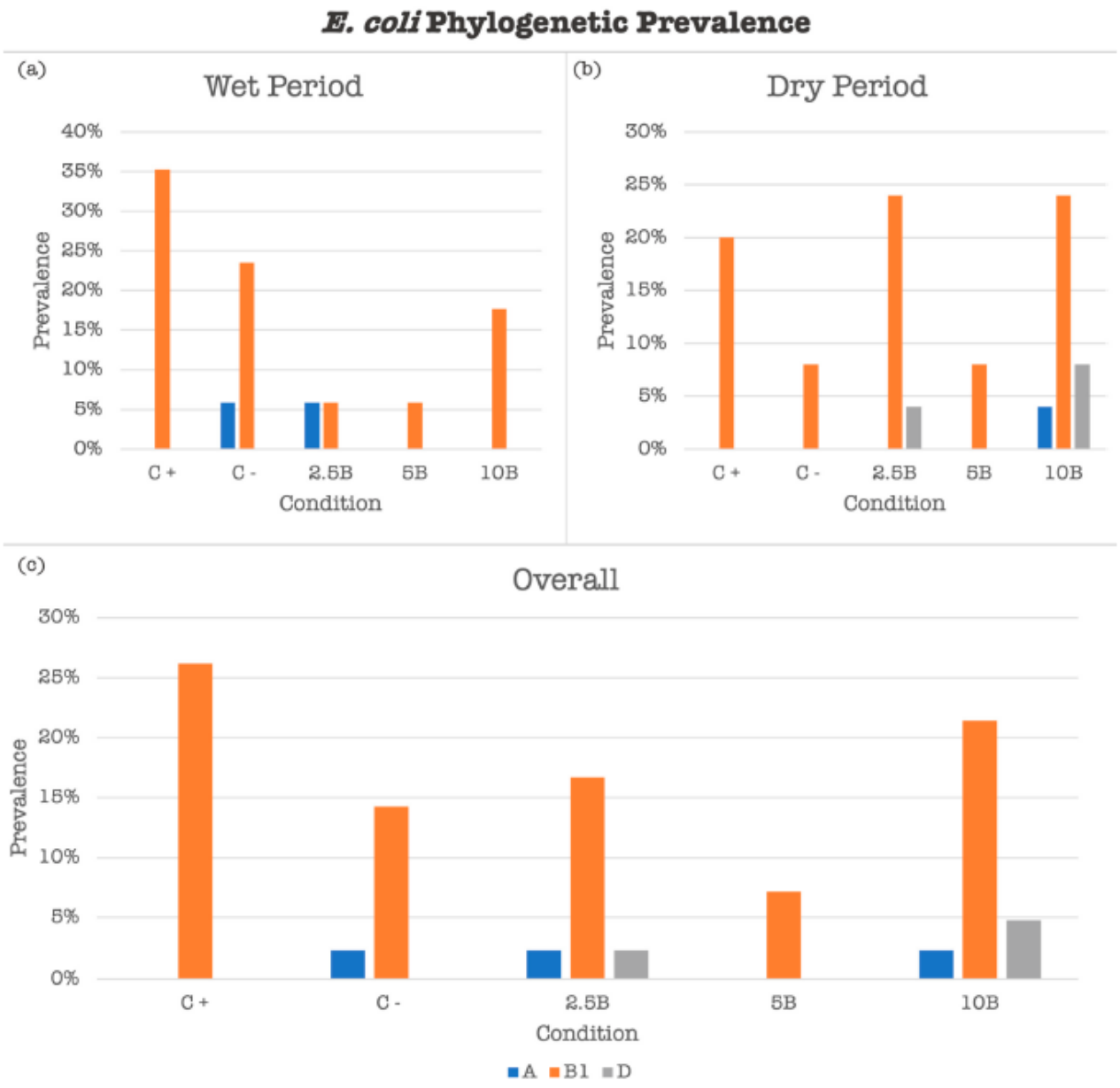


Figure 5 - The prevalence of *E. coli* phylogenetic groups. The prevalence of E. coli phylogenetic groups (A, B1, D) in RMS samples across different conditions and periods. (**a**) The prevalence of isolates obtained in the wet period (n = 17). (**b**) The prevalence of isolates obtained in the dry period (n = 25). (**c**) The overall resistance rates (n = 42). C+—positive control; C−—negative control; 2.5B—RSM supplemented with 2.5% biochar; 5B—RSM supplemented with 5% biochar; 10B—RSM supplemented with 10% biochar.

In the assay performed in the wet period (Figure 5a), isolates from the positive control (C+) condition exhibited the highest prevalence of phylogroup A (40%), followed by phylogroup B1 (5%). In contrast, RMS supplemented with 2.5% biochar (2.5B) had a notable presence of phylogroup B1 (5%), with that of phylogroup A remaining low (5%).

In the assay performed in the dry period (Figure 5b), RMS supplemented with 10% biochar (10B) showed a higher prevalence of phylogroup D (5%) compared to the control conditions. The 2.5% biochar condition also displayed a significant presence of phylogroup B1 (10%).
Overall, the prevalence of phylogroups (Figure 5c) indicates that the positive control (C+) and RMS supplemented with 10% biochar (10B) had the highest occurrences of phylogroup A (25% and 20%, respectively), with phylogroup D being more prevalent in the biochar-treated samples. However, these differences were not statistically significant.

3.4 Antimicrobial Susceptibility Testing

Table 9 presents the AMR profile of *Enterococcus* isolates from RMS supplemented with varying concentrations of biochar (2.5%, 5%, and 10%), alongside positive (C+) and negative (C-) controls, across different antibiotic classes.
The key findings indicate that for penicillins, resistance to ampicillin was the highest in the 10% biochar group (55%), followed by the 2.5% biochar group (30%), with no resistance being detected in the positive control. No resistance was observed for amoxicillin–clavulanic acid across all conditions. For glycopeptides, vancomycin resistance was 100% in the positive control, whereas the 10% biochar group showed the highest resistance (9%) among the supplemented groups. In the case of tetracyclines, the highest resistance to oxytetracycline was observed in the 5% biochar group (40%), with no resistance being detected in the negative and positive control groups. Aminoglycosides showed no resistance to high-dose gentamycin across all conditions. For fluoroquinolones, the highest resistance to enrofloxacin was observed in the 10% biochar group (36%), with no resistance being found in the positive control.
The enterococci from the RMS samples supplemented with 5% biochar exhibited the lowest overall resistance rates for this bacterial group, being primarily resistant to oxytetracycline (40%). However, the differences observed in the resistance rates demonstrated by these bacteria to various antibiotics were not statistically significant.

Table 9 **-** Results from the antimicrobial susceptibility tests for *Enterococcus* isolates (n_{total}=40). Isolates across all time points for each condition with corresponding susceptibility and resistance rates for each antibiotic tested. R (%): Percentage of resistant isolates; I (%): Percentage of intermediate isolates; S (%): Percentage of susceptible isolates. N/A – No breakpoints available.

		Penicillin's		**Glycopeptides**	**Tetracyclines**	**Aminoglycosides**	**Fluoroquinolones**
	Condition	**Ampicillin**	**Amoxicillin–Clavulanic Acid**	**Vancomycin**	**Oxytetracycline**	**High-Dose Gentamycin**	**Enrofloxacin**
%R	C+ (n = 3)	0	0	66.7	0	0	0
	C− (n = 11)	27.3	0	0	0	0	18.2
	2.5B (n = 10)	30	0	0	0	0	10
	5B (n = 5)	0	0	0	40	0	0
	10B (n = 11)	54.5	0	9.1	18.2	0	36.4
%I	C+ (n = 3)	N/A	0	33.3	0	0	100
	C− (n = 11)	N/A	0	9.1	18.2	0	36.4
	2.5B (n = 10)	N/A	0	40	10	0	50
	5B (n = 5)	N/A	0	20	0	0	0
	10B (n = 11)	N/A	0	9.1	18.2	0	9.1
%S	C+ (n = 3)	100	100	0	100	100	0
	C− (n = 11)	72.7	100	90.9	81.8	100	45.5
	2.5B (n = 10)	100	100	60	90	100	40
	5B (n = 5)	100	100	80	60	100	100
	10B (n = 11)	45.5	100	81.8	63.6	100	54.5

According to Table 10, the AMR profiles of *E. coli* isolates varied across the conditions tested. Regarding penicillins, resistance to ampicillin appeared to be higher in the isolates from the positive (36%) and negative controls (29%) when compared to those from the biochar-supplemented groups. In fact, 11% of the isolates from the samples supplemented with 2.5% biochar showed resistance to this antibiotic, while 8% of those from the samples supplemented with 10% biochar presented a resistance profile. Also, none of the isolates from the samples supplemented with 10% biochar were resistant to ampicillin. Additionally, no resistance to amoxicillin–clavulanic acid was observed in any of the conditions tested. However, the observed differences in resistance rates were not statistically significant.

Table 10 - Results from the antimicrobial susceptibility tests for *E. coli* (n_{total}=42). Isolates across all time points for each condition with corresponding susceptibility and resistance rates for each antibiotic tested. R (%): Percentage of resistant isolates; I (%): Percentage of intermediate isolates; S (%): Percentage of susceptible isolates. Bold results represent the highest %R for each antibiotic.

		Penicillin's		**Tetracyclines**	**Sulphonamides**	**Fluoroquinolones**	**Cephalosporins**
	Condition	**Ampicillin**	**Amoxicillin–Clavulanic Acid**	**Oxytetracycline**	**Trimethoprim–Sulfamethoxazole**	**Enrofloxacin**	**Ceftiofur**
%R	C+ (n = 11)	36.4	0.0	0.0	0.0	0.0	0.0
	C− (n = 7)	28.6	0.0	14.3	0.0	0.0	0.0
	2.5B (n = 9)	11.1	0.0	11.1	0.0	0.0	0.0
	5B (n = 3)	0.0	0.0	0.0	0.0	0.0	0.0
	10B (n = 12)	8.3	0.0	16.7	0.0	0.0	0.0
%I	C+ (n = 11)	36.4	0.0	0.0	0.0	0.0	0.0
	C− (n = 7)	28.6	14.3	14.3	14.3	0.0	0.0
	2.5B (n = 9)	33.3	0.0	0.0	0.0	0.0	11.1
	5B (n = 3)	33.3	0.0	0.0	0.0	0.0	33.3
	10B (n = 12)	41.7	0.0	0.0	0.0	0.0	0.0
%S	C+ (n = 11)	27.3	100.0	100.0	100.0	100.0	100.0
	C− (n = 7)	42.9	85.7	71.4	85.7	100.0	100.0
	2.5B (n = 9)	55.6	100.0	88.9	100.0	100.0	88.9
	5B (n = 3)	66.7	100.0	100.0	100.0	100.0	66.7
	10B (n = 12)	50.0	100.0	83.3	100.0	100.0	100.0

According to the classification presented by Magiorakos *et al.*, (2012), no isolate was considered as Multidrug Resistant (MDR) (Magiorakos et al., 2012)

3.5 Virulence Assays

Regarding enterococci's ability to produce virulence factors, biochar supplementation did not promote a reduction in virulence factors' production by RMS isolates, and the changes that occurred were not statistically significant (Table 11). For *Enterococcus* spp., hemolysin production was the highest in the positive control group (100%), followed by the 5% biochar group (80%). Biofilm production was the most prevalent in the 5% biochar group (60%), followed by the negative control group (45%). Proteinase production peaked in the 10% biochar group (82%). Gelatinase, DNase, and lecithinase were not detected in any of the conditions.

Table 11 - Virulence profile of the *Enterococcus* (n_{total}=40) and *E. coli* (n_{total}=42). Isolates across all time points for each condition. (P (%): Percentage of positive isolates; Bold results represent the highest %P for each virulence factor.

		Condition	Hemolysin	Gelatinase DNase Lecithinase	Biofilm	Proteinase
Enterococcus	%P	C+ (n=3)	**100**	0	0	0
		C- (n=11)	55	0	45	55
		2.5B (n=10)	60	0	40	30
		5B (n=5)	80	0	**60**	80
		10B (n=11)	73	0	36	**82**
E. coli	%P	C+ (n=11)	**100**	0	36	0
		C- (n=7)	**100**	0	**57**	0
		2.5B (n=9)	**100**	0	11	0
		5B (n=3)	**100**	0	0	0
		10B (n=12)	**100**	0	33	0

For *E. coli*, hemolysin production was uniformly high at 100% across all conditions. Biofilm production was the highest in the negative control group (57%), followed by the positive control group (36%). Gelatinase, DNase, proteinase, and lecithinase production were not detected in any conditions.

Table 12 presents the distribution of hemolysin production across different *Enterococcus* species isolated from RMS samples. *E. gallinarum* and *Enterococcus* sp. isolates exhibited the highest percentages of hemolysin production (33% and 30%, respectively), while lower percentages were observed for *E. faecalis* (11%), *E. faecium* (7%), and *E. hirae* (4%).

Table 12 - Hemolysin production (%) across Enterococcus species isolated from RMS samples.

	Hemolysin
E. faecalis	11%
E. faecium	7%
E. hirae	4%
E. gallinarium	33%
Enterococcus sp.	30%

A statistical analysis of antibiotic resistance and virulence factors was conducted to understand the impact of the bacterial group and biochar condition on these parameters. The results are summarized in Table 13.

Table 13 - A statistical analysis of antibiotic resistance and virulence factors. This table presents the *p*-values (significant *p*-value < 0.05) from the statistical analysis of the effects of the bacterial group and biochar condition on antibiotic resistance and virulence factors. Highlighted in gray are the results of the analysis performed for only one bacterial group. Highlighted in yellow are all isolates that presented the same phenotype. The dash (-) represents variables not included in the final models.

Antibiotic / Virulence Factor	Bacteria (*p*-value)	Condition (*p*-value)
Ampicillin	0.03	0.43
Oxytetracycline	-	0.80
Trimethoprim-Sulfamethoxazole		1.00
Enrofloxacin	-	0.92
Amoxicillin-clavulanic acid	-	1.00
Ceftiofur		0.95
Vancomycin		0.60
Gentamicin		-
Hemolysin	-	0.51
Gelatinase	-	-
Biofilm	-	0.63
DNase	-	-
Lecithinase	-	-
Proteinase	-	0.45

Regarding antibiotic resistance, the statistical analysis revealed that *E. coli* presented increased odds (3.10) of being resistant to ampicillin in comparison to enterococci. However, no significant differences were found regarding antimicrobial resistance profiles and different biochar supplementations ($p = 0.43$). No significant differences were found across any of the tested variables regarding resistance to other antibiotics.

Also, regarding virulence factors, no significant differences were found regarding any of the variables tested.

3.6 Isolates' Pathogenicity Potential – MAR and Virulence Indexes

The MAR and VIR indexes were calculated for each isolate of both *Enterococcus* spp. and *E. coli* across different biochar supplementation conditions. The average (AVG) and standard deviation (STD) values for each condition are summarized in Table 14.

Table 14 - MAR and VIR averages for each treatment condition. AVG – average; STD – standard deviation; Results in bold show values above cut-off. For each parameter, means with different superscript letters indicate significant differences between groups.

		C +		C -		2.5B		5B		10B	
		AVG	STD	AVG	STD	AVG	STD	AVG	STD	AVG	STD
Enterococcus	MAR	**0.33**	0.00	0.18	0.12	0.23	0.25	0.10	0.15	0.26	0.11
	VIR	0.17^{a}	0.00	0.26^{ab}	0.16	0.22^{a}	0.11	0.37^{b}	0.14	0.32^{b}	0.12
E. coli	MAR	0.12	0.08	0.19	0.24	0.11	0.08	0.11	0.10	0.11	0.08
	VIR	0.23	0.08	0.26	0.09	0.19	0.06	0.17	0.00	0.22	0.08

The MAR results were not statistically significant, while the VIR index showed a statistical trend for the relation between condition and bacteria (p = 0.10), since the isolates from the positive control and from the RMS supplemented with 2.5% biochar had a significantly lower VIR index than those from the RMS supplemented with 5% and 10% biochar. The MAR values indicate that *Enterococcus* isolates from the positive control exhibited the highest resistance, whereas isolates from the RMS supplemented with 5% biochar showed the lowest resistance. The VIR values for *Enterococcus* spp. were the highest amongst the isolates from the RMS supplemented with 5% biochar.

For *E. coli*, the highest MAR value was observed in the negative control, while the VIR values were relatively similar across different conditions, with the highest values being observed in the isolates from the negative control. The 5% biochar supplementation showed the lowest virulence index.

Moreover, only isolates from the positive control group met the criteria for being classified as a low threat, indicating a lower pathogenicity potential. In contrast, isolates from all other conditions were classified as posing no threat based on the averages of their MAR and virulence indexes.

4. Discussion

Understanding the dynamics of bacterial populations in RMS is crucial for improving animal and environmental health. Previous findings by Hutchison et al. (2005), Gurtler et al. (2018), and Rapp et al., (2023) demonstrate significant pathogen reductions in manure when using aerobic digestion processes (Gurtler et al., 2018; Hutchison et al., 2005; Rapp et al., 2023). The significant reduction in bacterial counts over time is likely due to aerobic digestion, which promotes the breakdown of organic matter, creating less favorable conditions for the survival of certain bacterial genera. Studies indicate that aerobic digestion, especially during the

thermophilic phase, plays a crucial role in reducing organic matter and pathogen load (Gurtler et al., 2018; Hutchison et al., 2005; Rapp et al., 2023). High temperatures during this phase favor the proliferation of thermophilic bacteria, such as those from the genera *Bacillus* and *Thermus*. These bacteria are essential in breaking down complex organic compounds, thereby reducing nutrient availability for other bacteria and creating an environment less conducive to their survival (Finore et al., 2023; Qian et al., 2016).

The initial degradation processes are carried out by mesophilic organisms. As the temperature rises due to the intense digestive activity of microorganisms, thermophilic populations take over and continue the conversion of organic compounds into carbon dioxide. This active stage of composting is characterized by rapid decomposition, persisting until the organic substrates are depleted. Subsequently, microbial activity declines, leading to a drop in temperature. During the curing phase, mesophilic organisms repopulate the compost, and humid substances accumulate, resulting in mature compost (Finore et al., 2023).

In our study, not all conditions tested promoted a reduction in the RMS' bacterial counts, and the decreases observed were not statistically significant. In comparison, (Mohammadi-Aragh, 2022) reported a 30% reduction in *E. coli* numbers, equivalent to a 1.79 Log10(CFU/g) reduction from a baseline of 5.98 Log10(CFU/g), when applying pine biochar to poultry manure.

The reductions obtained in the *Enterococcus* spp. counts are comparable to those obtained by Perez-Mercado et al. (2019), who reported a decrease of 4.4 Log10(CFU/g) in *Enterococcus* sp. when using biochar as a filter for farm wastewater (Perez-Mercado et al., 2019). This similarity highlights biochar's potential in reducing bacterial populations, although achieving statistical significance remains challenging.

A species diversity analysis provided insights into the microbial communities' composition and richness. The most predominant *Enterococcus* species detected were *E. hirae* and *E. gallinarum*. *E. hirae*, commonly found in plants and cattle, is an indicator of healthy animals because its presence is associated with normal, commensal gut microbiota in cattle, often isolated from bovine feces and manure (Messele et al., 2022). *E. hirae* pathogenicity is not as well characterized as for other enterococcal species; however, isolates from this species have been associated with infections mainly in humans, including pyelonephritis, endocarditis, and biliary tract infections (Zaidi et al., 2022). *E. gallinarum* is generally not associated with infections, but there are reports of this species presenting low-level resistance to vancomycin, which is concerning as vancomycin is often used as a last-resort antibiotic for treating serious infections caused by Gram-positive bacteria (Ramos et al., 2020; Willems et al., 2023). The emergence of vancomycin-resistant enterococci (VRE) can lead to limited treatment options and has significant public health implications. *Enterococcus* species with higher health risks, such as *E. faecium* and *E. faecalis*, were identified in the negative control and RMS

supplemented with 5% and 10% biochar. It is important to note that only four isolates from each condition and time point were selected for further characterization. This selection was carried out randomly, which may have influenced the variation in species identified in the control and treatment groups.

For *E. coli*, phylogenetic group D was observed exclusively in isolates from 2.5% and 10% biochar-supplemented RMS. Phylogenetic group D *E. coli* strains are particularly concerning because they are often associated with extraintestinal infections in humans, such as urinary tract infections, sepsis, and neonatal meningitis (Silva et al., 2009; Wang et al., 2023). Additionally, these strains can harbor multiple virulence factors and antibiotic resistance genes, making infections difficult to treat. In animals, phylogroup D *E. coli* can cause various diseases, including colibacillosis in poultry and mastitis in dairy cattle (Ghanbarpour & Salehi, 2010; Kolenda et al., 2015; Schouler et al., 2012). The presence of these potentially pathogenic strains in biochar-supplemented RMS highlights the need for a further evaluation of biochar's impact on microbial communities and its potential to influence pathogen prevalence.

The clonal analysis of both *Enterococcus* and *E. coli* isolates from RMS supplemented with biochar revealed some insights into the microbial community dynamics in dairy farming environments. Biochar supplementation does not appear to be crucial for the establishment of specific strains. Common to both bacterial groups is the presence of clones across different treatments and conditions, possibly representing the most persistent strains.

Overall, although no statistically significant differences were found between treatments, the variations between microbial populations suggest that biochar supplementation could influence the microbial community.

The structure of biochar provides a surface for microbial attachment, and its chemical properties could affect nutrient availability and microbial competition. In spite of the fact that the same batch of biochar was used in all assays, the different concentrations of biochar used for supplementation may have created micro-environments within the RMS that favored certain microbial species over others.

Analyzing bacterial AMR and virulence profiles offered deeper insights into the pathogenicity potential of these microorganisms. Enterococci from RMS samples supplemented with 5% biochar exhibited the lowest overall resistance rates, though resistance to oxytetracycline was notable at 40%. Similarly, *E. coli* showed intermediate resistance to ampicillin and ceftiofur at 33%. Studies have demonstrated that up to 20% of the administrated dose of oxytetracycline can be excreted by dairy cattle feces, which substantially contributes to its presence in manure (Oliver et al., 2020). These findings suggest that the high excretion rates of oxytetracycline may contribute to the resistance patterns observed, as the antibiotic's presence in manure provides selective pressure that promotes the development and persistence of resistant

bacterial populations (Richardson et al., 2018). This high excretion rate results in elevated concentrations of oxytetracycline residues in manure, making it one of the most prevalent resistances detected (Obaidat et al., 2018). However, while high excretion rates of oxytetracycline contribute to its presence in manure and may promote resistant bacteria populations, direct correlations between antibiotic use and resistance are complex and influenced by multiple factors, including environmental conditions, bacterial strain variability, and gene transfer mechanisms (Obaidat et al., 2018). Some resistance to other antibiotics was also observed, but none of the isolates met the criteria for MDR.

Other studies have demonstrated biochar's antimicrobial properties in various contexts (Chen et al., 2018; Cui et al., 2016; Du et al., 2023; Jang & Kan, 2022; Ma et al., 2024). For example, Jang and Kan (2022) found that biochar can effectively remove various ARGs, though some, such as *tetO* and *ermB*, may persist. Jauregi et al., (2023) reported that composting manure with 5% biochar increased the removal rate of specific ARGs, such as those encoding resistance to sulphonamides and tetracyclines (Jang & Kan, 2022; Jauregi et al., 2023). This aligns with our findings, particularly the absence of resistance to trimethoprim–sulfamethoxazole in any condition except an intermediate resistance found in the negative control.

While biochar's effects on soil microbial diversity and antibiotic resistance have been studied (Chen et al., 2018; Cui et al., 2016; Jang & Kan, 2022; Jauregi et al., 2023), its influence on bacterial virulence potential remains less understood. Our findings show that biochar supplementation did not significantly reduce the production of virulence factors by bacterial isolates. Yan et al. (2023) previously suggested that biochar might enhance microbial quorum sensing and biofilm formation, potentially improving cell viability and communication (Yan et al., 2023). For *Enterococcus*, only isolates from RMS samples supplemented with 2.5% biochar exhibited a lower virulence factor prevalence than those from the negative control, specifically regarding the production of biofilm (40%) and proteinase (30%). *E. coli* isolates from RMS supplemented with 5% biochar showed a lower virulence, with hemolysin being the only virulence factor detected. Regarding *Enterococcus* species, *E. gallinarum* and *Enterococcus* sp. exhibited the highest proportions of hemolysin-positive isolates, which might suggest a higher potential for pathogenicity. In contrast, *E. faecalis*, *E. faecium*, and *E. hirae*, although traditionally associated with high pathogenicity in clinical contexts, demonstrated significantly lower rates of hemolysin production. This finding might reflect species-specific variations in virulence factor expression or differences in environmental adaptation.

The pathogenic potential of bacterial isolates, assessed using MAR and virulence indexes, indicated that none of the isolates were classified as a high or moderate threat.

One important limitation of the present study is that it focused on two bacterial groups used as indicators for antimicrobial resistance dissemination, lacking a metagenomic approach. As

such, in the future, such an approach should be implemented on a larger number of samples collected at different seasons, aiming to better understand the effect of biochar supplementation on the resistome and virulome of RMS.

5. Conclusions

Considering animal and environmental health, this study aimed to evaluate the potential promising role of biochar in tackling AMR and virulent strains present in RMS. However, supplementation with different concentrations of biochar did not result in significant differences regarding bacterial loads or the presence of resistant and virulent bacteria in RMS.

The limitations of this study include its sampling design, with manure collected only twice—once in a wet period and once in a dry period—making it impossible to assess temporal or seasonal effects robustly. Additionally, the focus on *Enterococcus* spp. and *E. coli* limits the conclusions that can be drawn, and in the future, a metagenomic approach should be applied to provide a broader understanding of the influence of RMS supplementation with biochar, including in its resistome and virulome.

In conclusion, while the concentrations of biochar tested did not result in significant changes in bacterial loads nor the presence of resistant and virulent bacteria in RMS, this study provides an initial exploration of the potential of RMS supplementation with pine biochar. Further studies with larger sample sizes, expanded sampling points, and broader microbiological analyses are required to better evaluate biochar's role in improving RMS microbial safety, aiming at its application in agricultural systems.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/vetsci12010043/s1, Table S1: A BLAST comparison analysis of PCR product sequences obtained from *Enterococcus* isolates. The table lists the primers used, sequences obtained, product lengths, and the percentage identification for each sequence, confirming the species identification as *E. gallinarium*, Table S2: *Enterobacteriaceae* quantification in CFU/g and [Log10(CFU/g)] in MAC inoculated with samples collected during the assays performed in the dry and wet periods, Table S3: *Enterococcaceae* quantification in CFU/g and [Log10(CFU/g)] in SB inoculated with samples collected during the assays performed in the dry and wet periods.

Funding: This research was funded by CIISA—Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal, Project UIDB/00276/2020, and by the Associate Laboratory for Animal and Veterinary Sciences (AL4AnimalS), LA/P/0059/2020–AL4AnimalS (funded by FCT). The authors would also like to acknowledge the Foundation for Science and Technology for Ana Pires Ph.D. fellowship 2021.07175.BD)

Chapter III

Biochar supplementation affects the microbiome of recycled manure solids for cow bedding: a metagenomic analysis

---

Joana F. Guerreiro, **Ana José Pires**, Mónica Nunes, Ana Esteves, Lélia Chambel, Pedro Pascoal, Marcelo Pereira, David Fangueiro, Luís Tavares, Ricardo Dias, Ricardo Bexiga, Manuela Oliveira. 2024. Journal of Dairy Science. 10.3168/jds.2024-25616

Authors' contributions:

Joana F. Guerreiro: data analysis, drafting, critical revision, and final approval of the manuscript.

Ana Pires performed the experiments, help in drafting the manuscript.

Mónica Nunes: helped to perform the experiments.

Ana Esteves: helped to perform the experiments.

Lélia Chambel: helped in analysis of the data and manuscript revision.

Pedro Pascoal: helped in analysis of the data and manuscript revision.

Marcelo Pereira: helped in analysis of the data and manuscript revision.

Luís Tavares: contributed to manuscript revision.

David Fangueiro: contributed to the revision the manuscripts.

Ricardo Bexiga: helped to draft and revise the manuscripts and supervision throughout.

Manuela Oliveira: critical revision of the manuscripts, final manuscript approval and supervision throughout.

ABSTRACT

The widespread use of Recycled Manure Solids (RMS) as cow bedding material is not without risks, since cattle manure may act as a vehicle for pathogenic and antimicrobial resistant bacteria dissemination. Thus, our aim was to evaluate RMS-supplemented with a pine biochar produced in Portugal as a new cow bedding material, since the use of biochar has been shown to have the potential to mitigate the impact of relevant bacterial species when added to animal manure microbiota. Our experimental setup consisted on fresh RMS samples that were collected on a commercial dairy farm and placed in naturally-ventilated containers for a total of 4 groups: 1–non-supplemented RMS; 2-RMS supplemented with 2.5% (w/w) of biochar; 3-RMS supplemented with 5% (w/w) of biochar; and 4-RMS supplemented with 10% (w/w) of biochar. Sampling was performed at 4 different incubation times (0, 5, 15 and 30 days) and in two distinct seasons: April-May (humid season) and June-July (dry season). The resulting 32 samples were subjected to DNA extraction and their microbiome profile determined through complete 16S rDNA gene sequencing using Nanopore next-generation sequencing. We observed that biochar supplementation clearly altered the microbiome of RMS, which was reflected in changes in populations' diversity and their relative abundance of relevant pathogenic bacteria. In particular, we found that long-term storage (30 days) was more beneficial than short-term storage, an effect that was more evident for samples supplemented with 2.5% or 5% biochar. In both seasons, those concentrations of biochar led to a decrease in the levels of several mastitis-causing agents (Enterobacteriaceae, streptococci, enterococci and staphylococci). In addition, we also observed a reduction in the levels of *Salmonella* spp. and Gram-positive bacilli in the biochar-supplemented samples. Unexpectedly, however, those same conditions yielded an increase in the abundance of *Brucella* spp., a group which includes important infectious agents, highlighting the need for a deeper evaluation of the impact of biochar supplementation of RMS to ensure the future safe and sustainable use of this environmental-friendly resource in animal production.

Keywords: Biochar, Dairy cows, Microbial evaluation, Recycled Manure Solids

1.Introduction

Cattle manure contains high concentrations of bacteria (10^9 to 10^{10} CFU/g) and fresh manure, in particular, is associated with elevated counts of Enterobacteriaceae, *Staphylococcus aureus*, and *Enterococcus faecium*, making it a potential source of pathogens and antimicrobial-resistant bacteria and genes to humans, animals and the environment (Buta-Hubeny et al., 2022). The contamination of manure with relevant bacteria is especially important in farms affected by calf diarrhea, a multifactorial disease that involves several

pathogens, including *Salmonella enterica* and *Escherichia coli*, posing risks to both animal and human health (Naranjo-Lucena & Slowey, 2023).

Nevertheless, the reuse of cattle manure as bedding material for dairy cows in the form of Recycled Manure Solids (RMS) is becoming popular due to its cost efficiency and comfort when used as bedding material, offering a sustainable and easy to handle solution that aligns with modern farming practices (Jeppsson et al., 2024). The bedding material significantly impacts dairy cattle's health, as cattle spends much time lying down, allowing the contact between the animals' ventral region, namely the udder, and bedding bacteria. High counts of environmental pathogens in this material are a risk factor for the development of several infectious diseases in dairy cows (Rowbotham & Ruegg, 2016). One of those diseases is mastitis, a mammary gland inflammation often caused by microbial infection, which poses significant health and economic challenges to the dairy industry. Common pathogens associated with bovine mastitis include *Staphylococcus aureus*, non-*aureus Staphylococcus*, *Streptococcus* species (*S. agalactiae*, *S. uberis*, and *S. dysgalactiae*), and *E. coli* (Naranjo-Lucena & Slowey, 2023). Besides, dairy cows are also susceptible to reproductive tract inflammatory diseases, such as puerperal metritis, one of the reasons for antimicrobial treatment in dairy cattle. Metritis may be caused by multiple bacteria, including *E. coli*. *Brucella abortus* is another important agent associated with reproductive tract diseases in these animals, leading to abortion and birth of weak calves, posing a threat to livestock health and longevity as well as the sustainability of dairy farms. Brucellosis is also a relevant zoonotic disease (Khurana et al., 2021; Pires et al., 2024).

To mitigate the risk of RMS usage as cow bedding, several manure pretreatments are available. These include chemical treatments, physical methods, or biological processes, but they often lead to incomplete removal of antibiotic residues and pathogens (Varma et al., 2021). As such, to address the challenges associated with cattle manure management, the use of biochar, resulting from the controlled pyrolysis of organic materials, represents a promising alternative (Meyer et al., 2011). Biochar's high surface area and porous structure enable it to adsorb a wide range of substances, including antibiotics, also having a noteworthy impact on microbial communities within manure. Namely, biochar can promote the growth and activity of beneficial microorganisms while leading to the suppression of cattle pathogens and reducing disease transmission risks to humans (Ma et al., 2024).

In light of biochar's promising potential and the cost-effectiveness of RMS use as cow bedding, our study aimed to investigate the effects of biochar on the microbial community within RMS, focusing on potential cow pathogens such as Enterobacteriaceae, *Enterococcus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Salmonella* sp., and *Brucella* sp. Through a pilot incubation experiment conducted across two seasons, RMS obtained from a dairy farm was

supplemented with different biochar concentrations, and the microbiome's dynamics was analyzed post-incubation.

2. Materials And Methods

2.1 Sample Collection and Processing

This research was carried out using fresh RMS from a commercial dairy farm located in the south of Portugal, obtained through mechanical separation from fresh slurry (liquid manure) by a screw mechanism. A pilot incubation experiment was set up during two distinct time periods: the humid season (April-May 2022) and the dry season (June-July 2022). For that, samples comprising 5 kg of RMS were placed in naturally ventilated containers, which were assigned to four different groups containing: 1) non-supplemented RMS (negative control); 2) RMS supplemented with 2.5% biochar (w/w); 3) RMS with 5% biochar (w/w); and 4) RMS with 10% biochar (w/w). After incubation at ambient temperature for four distinct time periods (0, 5, 15 and 30 days of incubation), 10 g of RMS from each of the three replicate containers set per condition were collected, treated as a composite sample and stored at -20ºC to be used in subsequent analyses. Overall, 16 composite samples were obtained per season, for a total of 32 samples for both seasons under study.

2.2 DNA extraction

The 32 samples to be analyzed were subjected to DNA extraction, which was carried out using the DNeasy PowerMax Soil Kit (QIAGEN, Venlo, the Netherlands), following the manufacturer's instructions. DNA's quality and concentration were assessed by NanoDrop One and Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., Waltham, USA).

2.3 Amplification of 16S rDNA gene and Microbial Diversity Profiling through Next-Generation Sequencing

For the rDNA gene amplification Long Amp hot start Taq 2× master mix (New England Biolabs, MA, USA) was used at 1X along with 50 ng/µL of genomic DNA from each sample. To amplify the full-length 16S rDNA bacterial gene, 0.25 µM of the primer pair 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) were used. The PCRs were conducted on a Biometra UNO II, using the following conditions: 1 cycle of 94°C for 1 min, 35 cycles of 94°C for 20 s, 55°C for 30 s, and 65°C for 2 min, and a final extension of 65°C for 5 min.

Subsequently, amplification products were visualized through gel electrophoresis and purified using the Solid Phase Reversible Immobilization (SPRI) technique with magnetic beads (DeAngelis et al., 1995; Stortchevoi et al., 2020).

Quantification steps were performed using the 1xdsDNA HS assay for Qubit. DNA was end-repaired (New England BioLabs, MA, USA), cleaned with Agencourt AMPure XP Beads (Beckman Coulter, High Wycombe, UK) and dA-tailed (New England BioLabs, MA, USA). The library was prepared from 300 ng input DNA from each sample using the Sequencing Native Barcoding Kit 24 V14 (SQK-NBD114.24) (Oxford Nanopore Technologies, Oxford, UK) in accordance with the manufacturer's protocol. The library was quantified and prepared for PromethION sequencing, using FLO-PRO114M flowcells, MinKNOW v22.12.4, standard 72 h run script with active channel selection enabled. After 24h yielded 3.5 million passed reads with an estimated N50 of 1500bp and the mean quality score was 14.5. In total 5.25 Gb of data were produced, with an average of 110,000 reads per sample.

2.4 Bioinformatics and Statistical analyses

The sequencing data obtained from 16S amplicons was initially preprocessed, to ensure the accuracy and reliability of the results obtained. Specifically, low quality reads were removed and only the remaining reads with lengths higher than 200 bps were retained using the Prinseq-lite tool. Moreover, reads with a Q score below 7 were also disregarded (Schmieder & Edwards, 2011). Taxonomic classification followed a Lowest Common Ancestor approach and was performed through indexing based on k-mers mapping to the lowest common ancestor of all genomes known to contain a given k-mer (Wood et al., 2019). This classification used as reference databases the NCBI RefSeq reference genomes and NCBI GenBank reference sequences of Archaea and Bacteria (up to May 2023).

Following classification, data was rarefied and subjected to: Shannon diversity index analysis (McMurdie & Holmes, 2013); alpha diversity group significance analysis (Bolyen et al., 2018); and sample dissimilarity analysis – Principal Coordinates Analysis (PCoA) for beta diversity analysis based on the Bray-Curtis similarity index and determination of taxa abundance (with a genera prevalence cutoff of ≥ 0.01) (Bolyen et al., 2018). Prior to Shannon diversity index analysis, to account for uneven sampling depth, the data were rarefied to the minimum sampling depth of 8000 sequences. To produce the alpha diversity graphics (for both Shannon and Pielou's evenness indexes), the samples were randomly subsampled to create 3 technical replicates. Statistical analysis was then performed using the Kruskal-Wallis test for pairwise comparisons between each sample and the respective negative control. Significant differences were considered when the p-value (P) < 0.05. Beta diversity was evaluated by PCoA based on Bray-Curtis Index distance using QIIME (Bolyen et al., 2018). Only families/genera corresponding to a relative abundance higher than 0.1% were considered for analysis (Cunha et al., 2021)

3. Results

3.1 Bacterial Populations in Biochar-supplemented Recycled Manure Solids

The results from Next-generation sequencing and subsequent taxonomical analyses revealed a distribution of each level that corresponded to a total of 23 phyla, 41 classes, 85 orders, 195 families, 467 genera and 862 species for the samples collected in the humid season and a total of 48 phyla, 82 classes, 166 orders, 358 families, 1001 genera and 2117 species for the samples collected in the dry season (Supplemental Tables S1 and S2; https://doi.org/10.7910/DVN/JFOSKC). These results evidenced a clear difference in taxonomic richness between the samples collected in both seasons, with the samples of the dry season having twice the number of all taxonomic levels on average when compared with the samples of the humid season. This provided us with a first indication that the samples of the dry season had a more complex bacterial population than the one found in the humid season samples.

The beta diversity analysis shown in Figure 6 allows us to observe that for both seasons the initial samples collected were similar but started to diverge with time. However, that evolution in microbial populations seemed to progress faster in the dry season than in the humid season, as evidenced by the fact that the samples collected on days 15 and 30 in the dry season were much more similar among themselves than those from the humid season. Seeing that population dynamics tended to stabilize only for the longer incubation period, moving forward we will mostly focus on the data obtained for those time points (day 30).

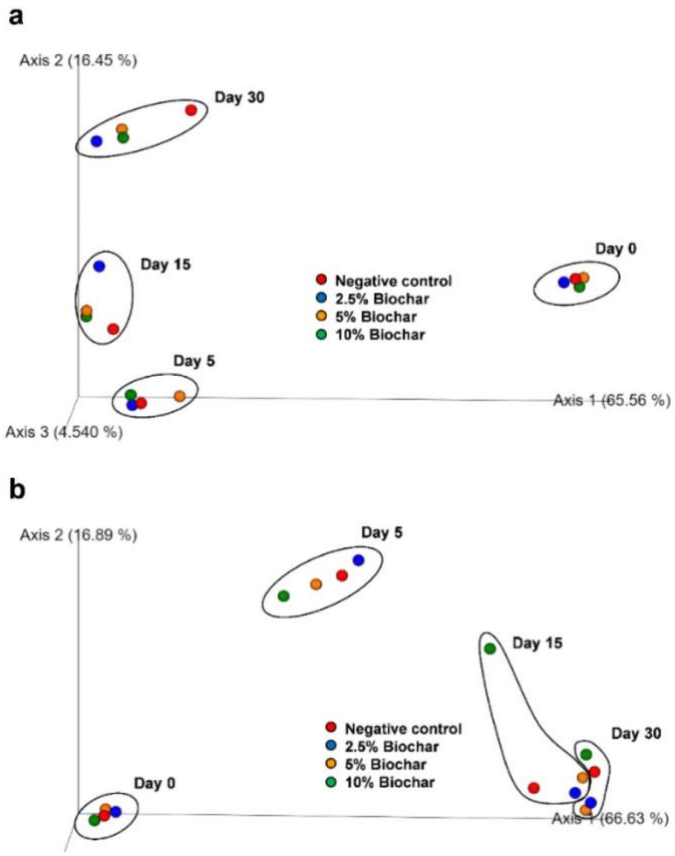


Figure 6 - Principal Component Analysis (PCA) of bacterial communities in the humid (a) and dry (b) seasons at the different times of incubation and under all the conditions tested in this study.

Next, we assessed the alpha diversity of the samples under study, for both seasons, using Pielou (Figure 7a and c; Supplemental Tables S3 and S4; https://doi.org/10.7910/DVN/JFOSKC) and Shannon indexes (Figure 7b and d; Supplemental Tables S3 and S4), which assess the evenness of the distribution and the overall diversity of microbial types within a population, respectively. After 30 days of incubation during the humid season, the average Pielou index of the negative control was 0.86, while that of biochar-supplemented samples was 0.82, 0.81 and 0.82 for the samples with 2.5%, 5% and 10% of biochar, respectively. The corresponding values of those samples for the Shannon index were 7.95, 7.24, 7.37 and 7.52. Therefore, biochar addition led to a statistically significant decrease (p-value = 0.0495) in both the evenness and diversity of the microbial populations present in the biochar-supplemented samples. We observed the exact same effect in the biochar-supplemented samples in the dry season, except for the 10% biochar-supplemented sample that presented a significantly lower evenness (p-value = 0.0495) than the negative control but exhibited no significant differences in the Shannon index (p-value = 0.512). In general, biochar-supplementation had a significant impact in the populations' alpha diversity, leading to lower species richness in those samples. Remarkably, the results obtained also pointed to an overall higher diversity in the dry season, since the Shannon index was found to be, on average for the four samples analyzed at 30 days of incubation, about 8% higher in the dry

season than in the humid season (values of 8.09 and 7.52, respectively), even though the Pielou index exhibited a slight decrease (of approximately 5%). This is in accordance with the higher number of taxonomic levels mentioned above (Supplemental Tables S1 and S2), which had already suggested the existence of more diverse bacterial populations in the dry season.

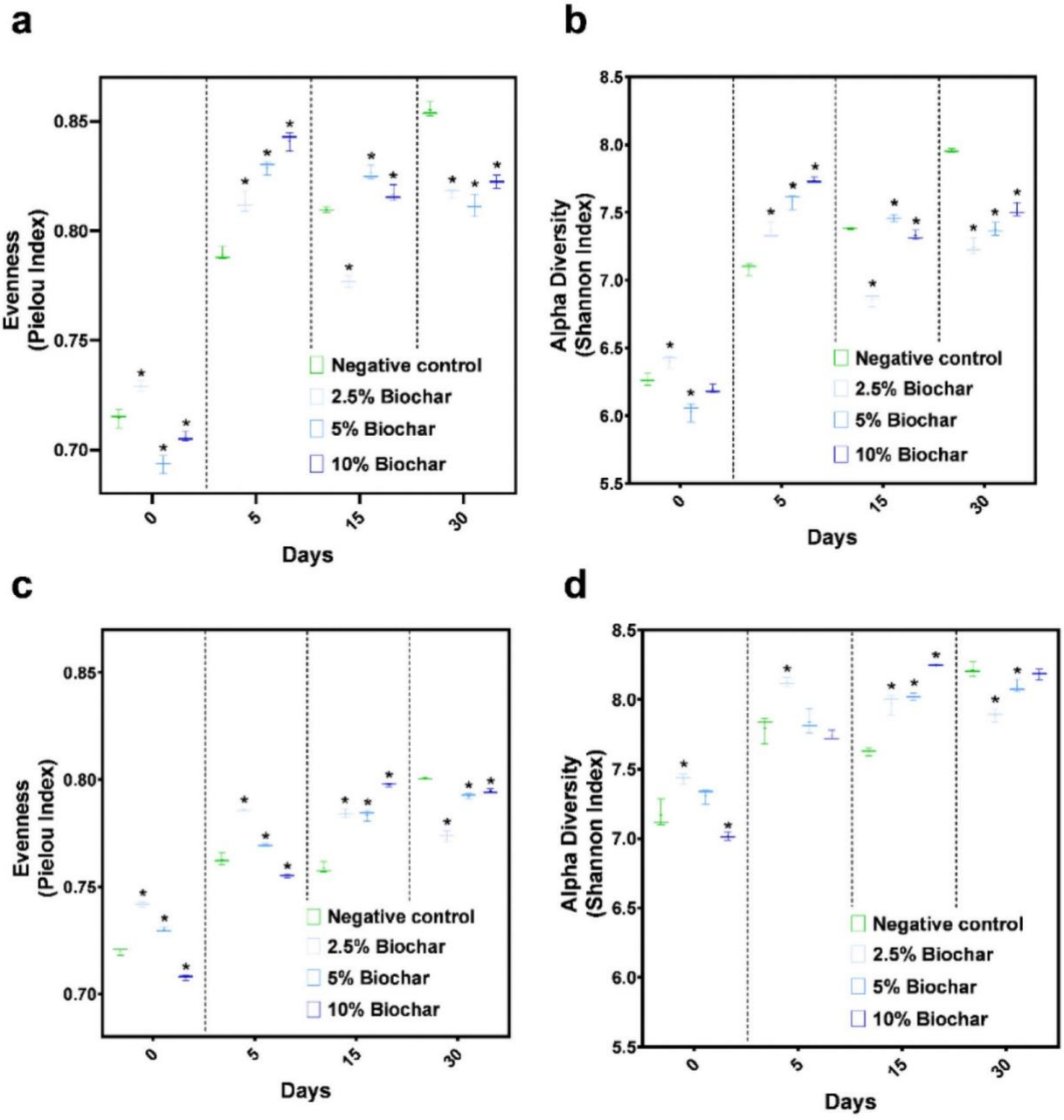


Figure 7 - Alpha diversity boxplots of bacterial communities in the humid (a, b) and dry (c, d) seasons at the different times of incubation and under all the conditions tested in this study. Bacterial evenness estimated by the Pielou index (a, c) and bacterial diversity estimated by the Shannon index (b, d) in the humid and dry seasons, respectively. Statistical analysis was performed using the Kruskal–Wallis test and a significant p-value is represented by an asterisk (*p-value <0.05).

3.2 Main Causative Agents of Bovine Mastitis Present in RMS

Considering our goal of evaluating whether biochar supplementation could promote changes in the pathogenic RMS microbiome, we initially set out to assess the relative abundance of the most common causative agents of bovine mastitis in the RMS samples. We detected the presence of the most relevant genera known to cause mastitis in cows in all the samples, including members of the Escherichia, Streptococcus, Enterococcus, and Staphylococcus genera. Remarkably, the relative abundance of all these genera was found to decrease at 30 days of incubation, in both seasons, in the biochar-supplemented samples, particularly those containing 2.5% and 5% of biochar. In the humid season, where the differences were more pronounced, the percentage of reduction in the samples containing 2.5% and 5% of biochar, when compared with the negative control, was as follows (respectively): 29.71% and 41.48% in members of the Enterobacteriaceae family, with a 53.55% and 68.96% decrease in Escherichia spp.; 7.1% and 53.44% in species belonging to the Enterococcus and Streptococcus genera; and 83.61% and 83.57% in staphylococci (Figure 8; Supplemental Tables S5 and S6; https://doi.org/10.7910/DVN/JFOSKC).

In the dry season, the decrease observed in the relative abundance of these genera in the equivalent samples (with 2.5% and 5% biochar) was: 39.82% and 25.51% in Enterobacteriaceae, and 42.88% and 22.54% in Escherichia spp.; 18.76% and 33.21% in enterococci and streptococci; and 65.28% and 66.37% in staphylococci (Figure 9; Supplemental Tables S5 and S6).

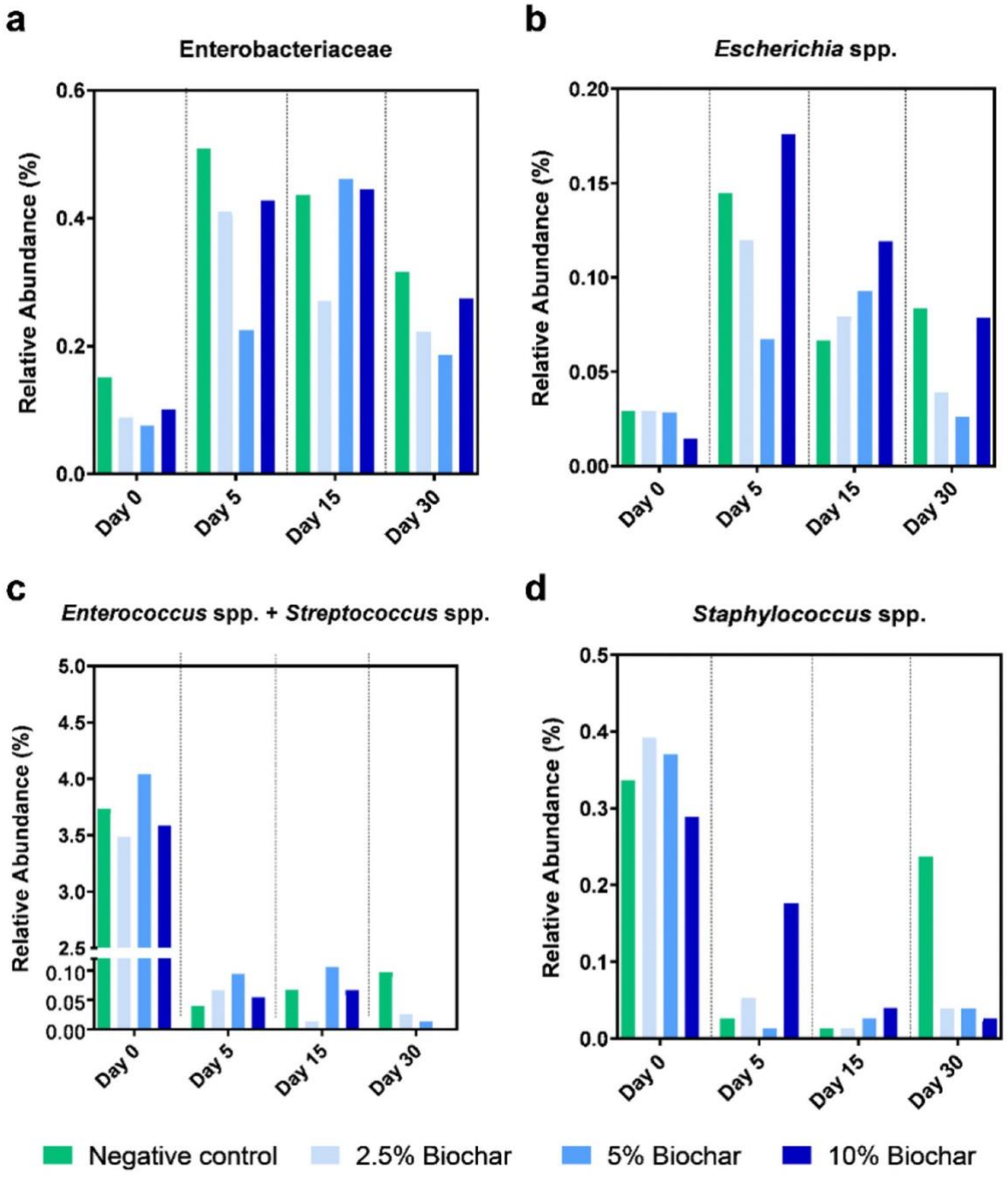


Figure 8 - Relative abundance of selected families (a) and genera (b, c, d) found in the samples collected in the humid season, which are relevant to the development of bovine mastitis.

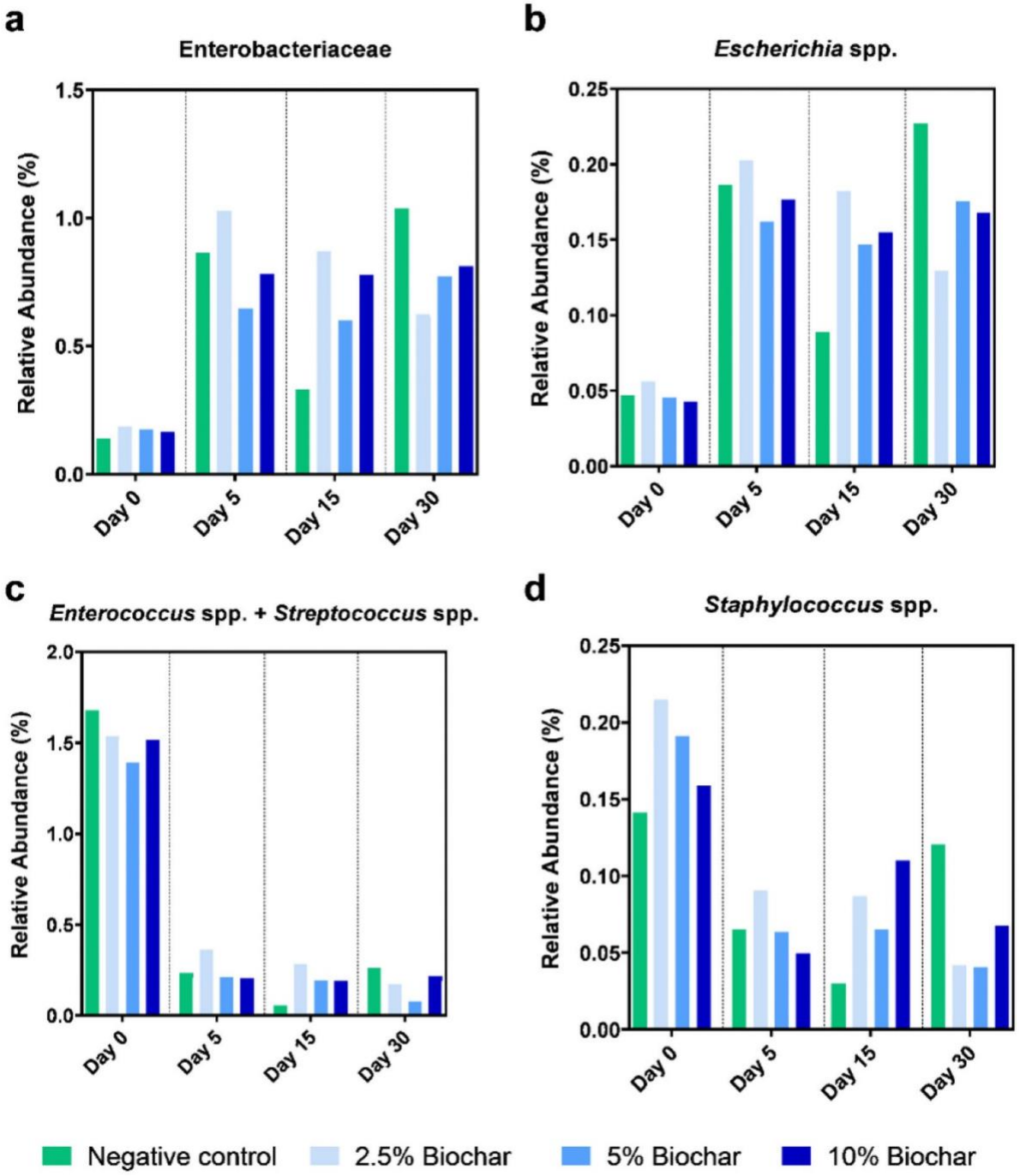


Figure 9 - Relative abundance of selected families (a) and genera (b, c, d) found in the samples collected in the dry season, which are relevant to the development of bovine mastitis.

3.3 Other Relevant Agents from a One Health Perspective Present in RMS

Since the use of RMS as cow bedding has implications that go beyond the scope of bovine health, presenting a potential environmental and human threat, we also focused the analysis on bacterial genera that are relevant from a One Health perspective. In that context, one discriminative difference observed was the marked reduction observed in the relative

abundance of spore-forming Gram-positive bacilli in biochar-supplemented RMS samples (Figures 10a and 11a; Supplemental Tables S5 and S6).

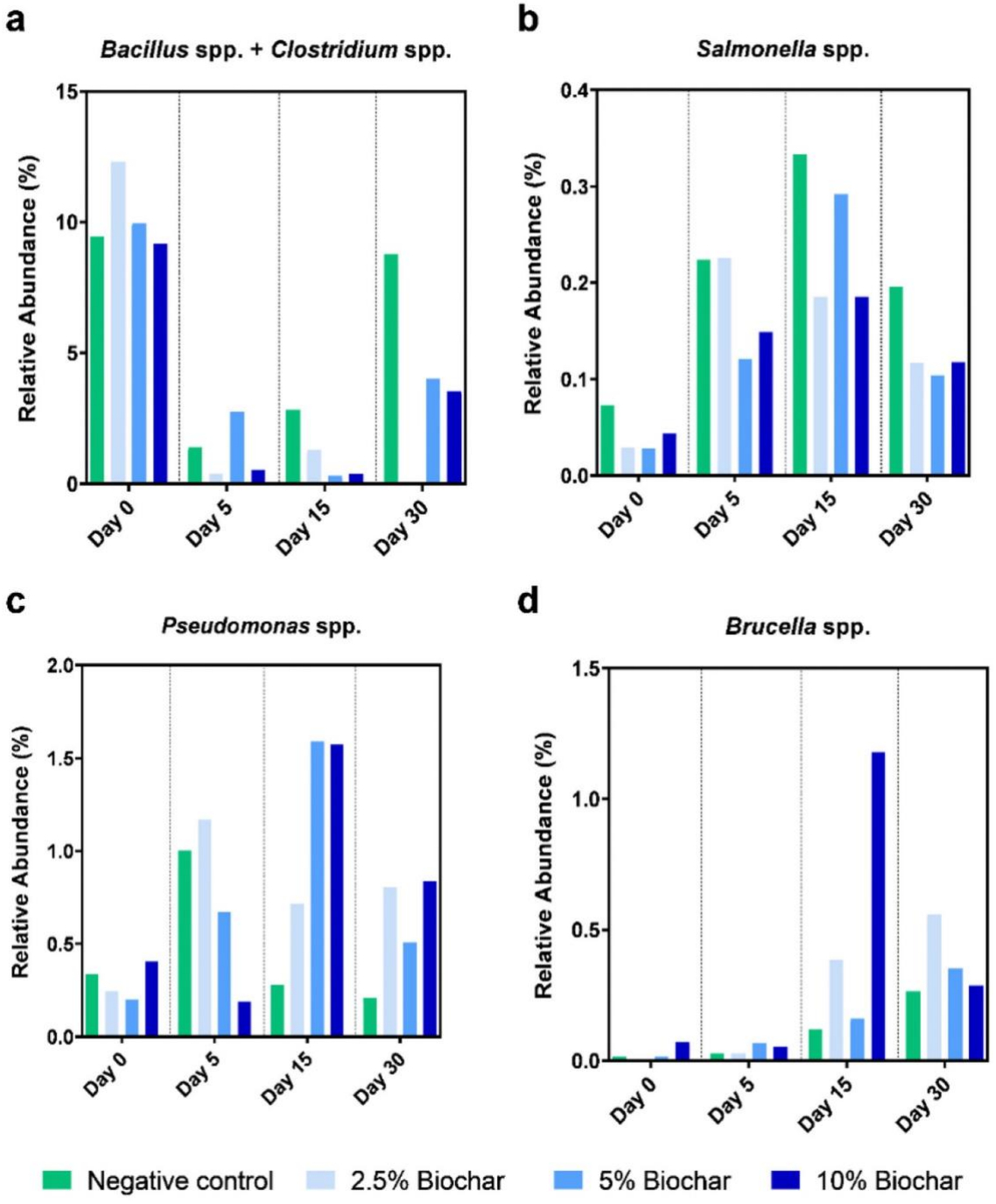


Figure 10 - Relative abundance of selected genera found in the samples collected in the humid season, which are relevant in a One Health perspective.

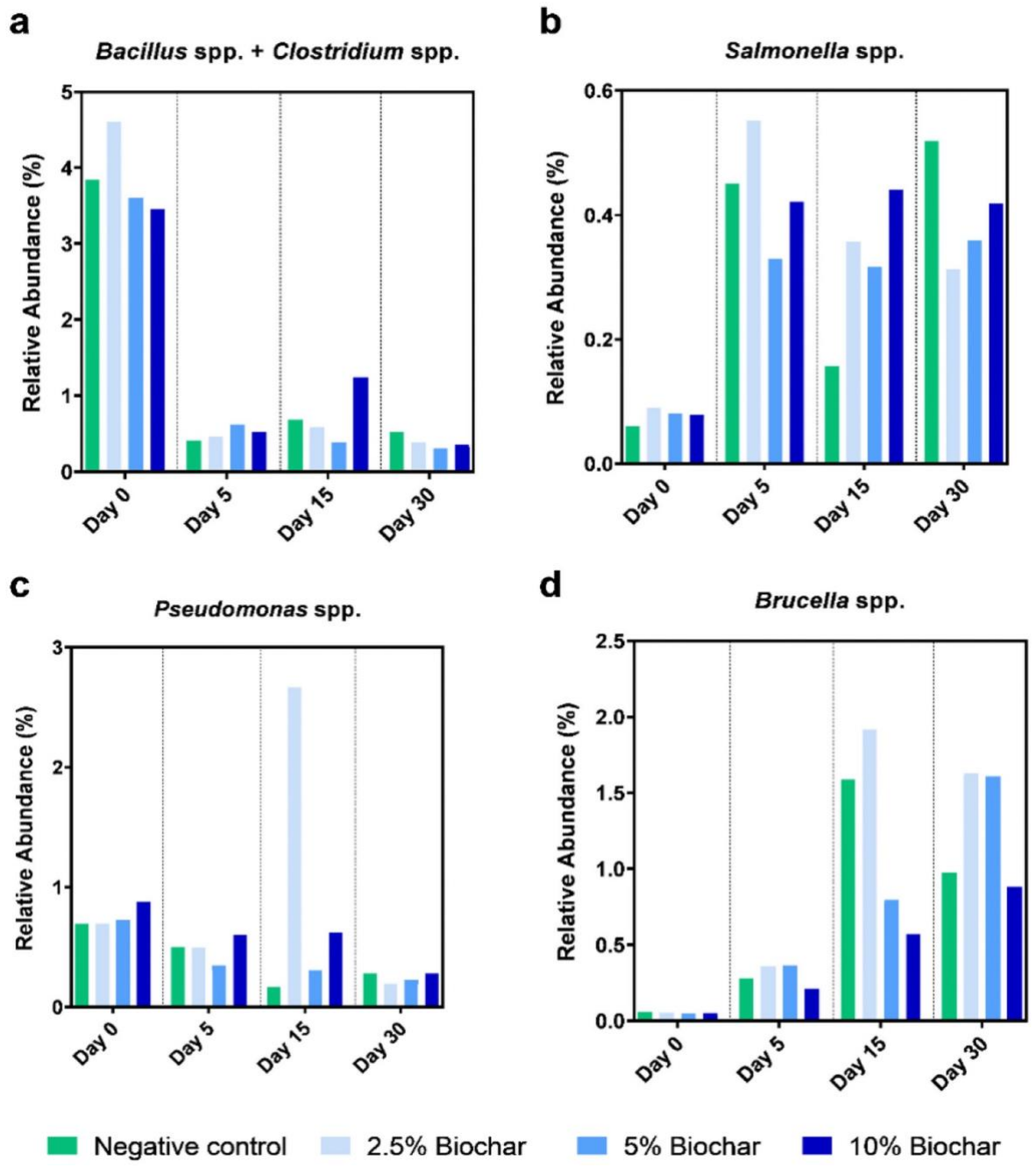


Figure 11 - Relative abundance of selected genera found in the samples collected in the dry season, which are relevant in a One Health perspective.

Once more, this divergence was more evident in the humid season, with the combined relative abundance of members of the Bacillus and Clostridium genera exhibiting a decrease of 99.58%, 55.42% and 60.32% in the 2.5%, 5% and 10% biochar-supplemented samples, respectively, when compared with the negative control. While more modest, an overall decrease of 26.52% was also observed for the three biochar-supplemented samples from the dry season. Additionally, one other pathogen, relevant in terms of gastrointestinal health in both humans and cattle, was found to have decreased abundance in the samples where biochar had been added. Salmonella spp. were found to be, on average, 42.27% and 29.98%

less abundant in the biochar-supplemented samples than in the control sample, in the humid and dry seasons, respectively (Figures 10b and 11b; Supplemental Tables S5 and S6). This was particularly relevant since Salmonella was the most abundant genus belonging to the Enterobacteriaceae family detected in this study in samples from both seasons.

Despite the promising results mentioned so far, not all changes observed in biochar-supplemented samples were desirable. Unexpectedly, we observed an increase in the relative abundance of two known bovine pathogens, which are also relevant pathogens in other species, including humans. Specifically, in the humid season, members of the Pseudomonas and Brucella genera were found to have a very noteworthy upsurge in biochar-supplemented samples, particularly in the sample supplemented with 2.5% biochar (Figure 10c and d; Supplemental Tables S3 and S4), where they rose 283.99% and 110.25%, respectively, in relation to the negative control. In the dry season, the relative abundance of Pseudomonas spp. showed the opposite trend, decreasing 31.15% in the 2.5% biochar-supplemented sample, but the levels of Brucella spp. remained much higher (by 67.06%) than those of the negative control (Figure 11c and d; Supplemental Tables S5 and S6).

4. Discussion

This study was carried out to elucidate the potential of biochar supplementation in increasing the safety of RMS used as bedding material for dairy cows. The sustainable aspect of this work focused not only on reusing manure solids as a cost-effective and environmentally-friendly bedding strategy, but also on the use of a pine biochar produced locally in Portugal. However, prior to being used as bedding material, RMS have to comply with microbiological requirements. In that context, we collected RMS samples, which were then treated (or not, as a control) with different concentrations of biochar and incubated at ambient temperature for up to 30 days, before we characterized their microbiome using next-generation sequencing. The data obtained revealed a complex series of changes in the bacterial dynamics of biochar-supplemented samples that shed some light on the potential benefits, but also on some limitations, of the use of this new bedding material.

The first objective was to evaluate the presence of several important mastitis-causing pathogens in RMS samples and whether biochar could play a role in reducing the levels of those pathogens. This is particularly relevant in the case of RMS-based bedding materials, since it has previously been shown that even though the bacterial load present in composted RMS decreases when compared with fresh RMS (namely that of coliforms and *Streptococcus* spp.), several mastitis pathogens are not eradicated, maintaining bacterial counts that can be problematic (Cole & Hogan, 2016). In this study, we did observe a decrease in the levels of *Streptococcus/Enterococcus* and *Staphylococcus* throughout time, but not in the Enterobacteriaceae level.

Moreover, fresh manure solids have been demonstrated to have higher total bacterial numbers before use than other fresh bedding materials, such as sand, recycled sand, paper fiber and straw (Alanis et al., 2021; Bonhotal et al., 2010). While average bacterial counts generally increase for all bedding types after use, used manure solids were found to have higher levels of streptococci than used and recycled sand; of coliforms than used paper fiber, sand and recycled sand; and of non-coliforms than all those types of beddings after use (Alanis et al., 2021). According to our data, we were unable to completely eradicate these pathogens in RMS using biochar supplementation, since we detected the presence of several members of the *Escherichia*, *Streptococcus*, *Enterococcus,* and *Staphylococcus* genera, even in samples that were incubated with biochar for as long as 30 days (the exception being the 10% biochar-supplemented sample in the humid season in which no enterococci or streptococci were detected). In fact, it would be very difficult to eliminate these pathogens using this type of additive, and the use of more aggressive methods could compromise the subsequent use of RMS as cow bedding. As such, it is particularly encouraging that, in this work, we observed a very marked decrease in the level of all of those previously mentioned pathogens. In the humid season, the most beneficial effect was observed for the samples supplemented with 5% of biochar, which led to an average reduction of 59.50% of Enterobacteriaceae, streptococci, enterococci and staphylococci as a whole. In the dry season, the supplementation with 2.5% or 5% of biochar had similar effects, leading to an overall decrease of these four groups by 41.29% or 41.70%, respectively. The main difference between the use of these concentrations was that 2.5% of biochar led to a higher reduction in Enterobacteriaceae levels, while 5% of biochar was more effective in reducing the levels of streptococci/enterococci. This is in agreement with a report from other authors that has shown that pine biochar application to poultry litter led to a significant reduction in *E. coli* and total aerobic bacteria counts (Mohammadi-Aragh, 2022). In most of the scenarios analysed, the use of a higher concentration of biochar (10%) didn't seem to yield better results than the use of lower concentrations (2.5% and 5%), demonstrating that the best overall performance could be obtained using the most cost-effective hypotheses.

Interestingly, previous research has reported that some strategies, such as replacing RMS daily from the back one-third of cow stalls, reduced cow's exposure to coliforms, but was ineffective against *Streptococcus* spp. (Sorter et al., 2014). Since composted RMS has been shown to have reduced bacterial counts before use, but that effect is soon lost as bacterial counts rise drastically after use, the emphasis should probably be put in the management of bedding once in use (Bonhotal et al., 2010; Sorter et al., 2014). In that sense, biochar might represent an advantage when added to RMS before use, but mainly during its usage in stalls, as it can decrease the levels of relevant pathogens on site, which seems to be the main factor needed to protect the udder from exposure to mastitis-causing pathogens.

It has been shown by many authors that there is a clear positive correlation between the dry mater bedding content and the growth of *Streptococcus* spp., coliforms, and non-coliforms and incidence of environmental clinical mastitis (Alanis et al., 2021; Fávero et al., 2015; Freu et al., 2023). Seeing that bed moisture plays such an important role in the development of mastitis, we performed our pilot experiment in two different seasons, to account for the effect of seasonality on the effectiveness of biochar's supplementation of RMS. Remarkably, we observed the largest decrease in causative agents of mastitis in the humid season, which is fairly promising since this is the season in which keeping the bedding drier represents a bigger challenge, as the relative environmental humidity was much higher (71.5%) than in the dry season (56.3%). We also observed that dry season samples presented a higher microbial diversity than the humid season samples, which could be related with the fact that it was in the dry season that we observed the highest overall variation in both temperature and humidity (of 12.6ºC and 56.0% as opposed to 9.5ºC and 43.0% variation in the humid season). It is possible that biochar could be overall more effective in the humid season, decreasing or increasing more drastically the levels of other bacteria in the community, thus leading to less diverse communities with less evenly distributed genera. Consistent with a previous report that assessed the effect of biochar addition on cow manure composting, we also observed that biochar supplementation led to a decrease in the diversity of the microbial populations under study (Ma et al. 2024).

Two additional positive effects of biochar addition to RMS observed in our work were the decreased levels of *Salmonella* spp. and Gram-positive bacilli found in supplemented samples, evidencing an interesting additional effect of biochar in reducing the level of known foodborne pathogens or spore-forming bacteria that can constitute a hazard even in pasteurized milk. While previous studies have determined that the use of RMS bedding did not lead to an increased presence of *Salmonella* spp., *Bacillus cereus* or bacterial spores in milk, those pathogenic agents were still found to be present in a small amount of samples (Bradley et al., 2018; Gagnon et al., 2020). Considering the risk that these pathogens of zoonotic interest may pose, in particular when one considers the ability of bacterial spores to resist high temperatures, including the ones used in milk pasteurization, it is particularly encouraging that biochar exhibited such an effective result in the reduction of these bacterial genera. Use of RMS bedding was also reported to increase the risk of detecting thermoresistant streptococci and enterococci in milk, which could have an important impact in the food industry, for instance by affecting the organoleptic properties of cheese (Gagnon et al., 2020). In this context, biochar can also help to prevent or minimize that risk, since it displayed a good efficacy in the reduction of the levels of streptococci and enterococci in RMS, according to the results obtained in our study.

Despite all the promising insights into the potentially beneficial role of biochar addition to RMS discussed so far, our study also revealed some undesirable effects. The most significant was the very marked increase observed in the levels of *Brucella* spp. in biochar-supplemented samples. Albeit surprising, this stimulating effect of biochar-like substances on *Brucella* growth had already been described as early as in 1951, when *Brucella suis* was cultivated in a system based on charcoal and cellophane, and activated charcoal continues to be used in the development of growth medium selective for *Brucella* (Gorelick et al., 1951; Mena-Bueno et al., 2022). At the time of collection of the RMS samples used in this study, we had no knowledge that *Brucella* spp. were circulating in the dairy farm in question. However, a brucellosis control program performed later in the year, in November 2022, led to a few animals testing positive for brucellosis. Considering the health and economic constraints this disease represents for a dairy farm, routinely screening cow bedding for the presence of specific pathogens might present an opportunity for early detection and subsequent prevention of higher losses.

The second troublesome effect we observed after biochar addition was an increase in the relative abundance of members of the *Pseudomonas* genus in the humid season, which includes species that are relevant bovine and human pathogens, such as *P. aeruginosa*. However, in the dry season, the opposite effect was observed. This is somewhat in agreement with what is found in the literature, where conflicting effects have been reported in this matter. While a recent study has shown that biochar addition to cow manure promoted its maturity through the reduction of the abundance of *Pseudomonas* spp., another study suggested that biochar may promote quorum sensing and biofilm formation in *P. aeruginosa*, which would make them thrive and be harder to eliminate from the environment (Ma et al., 2024; Yan et al., 2023). Further studies are thus needed to understand these dynamics and the factors that play a role in this process, even though the seasonality of this event observed in our study might indicate that humidity or temperature might be crucial. In addition, overall, these results highlight that while RMS supplementation with biochar can be an effective way to reduce the level of some relevant pathogenic species, its use should be further investigated, in particular to account for off target, undesirable effects that can compromise its efficacy and safety.

One important limitation of the present study is that the metagenomic analysis performed detects environmental DNA and not the pathogens directly. As such, in the future, it would be important to further investigate the potential of biochar in modulating the level of relevant pathogens, both in supplemented and non-supplemented manure samples, and in milk samples of animals with mastitis, in order to better understand the potential benefits of the findings described herein, namely regarding a useful effect of biochar on decreasing exposure to mastitis-causing pathogens to the dairy farm from which the RMS samples were sourced.

NOTES

This work was supported by the CIISA/FMV Project UIDB/00276/2020, LA/P/0059/2020—AL4AnimalS and the LEAF project UIDB/04129/2020 and UIDP/04129/2020, all funded by FCT. We also acknowledge Instituto Português do Mar e da Atmosfera (IPMA) that provided us with the weather data for the periods in which our incubation experiments took place.

All animals were cared for according to the rules given by the current EU (Directive 2010/63/EC) and national (DL 113/2013) legislation and by the competent authority (Direção Geral de Alimentação e Veterinária, DGAV, www.dgv.min-agricultura.pt/portal/page/portal/DGV).

Only noninvasive samples were collected during routine procedures with consent of owners, and no ethics committee approval was needed. Trained veterinarians obtained all the samples, following standard routine procedures. No animal experiment has been performed in the scope of this research. Verbal informed consent was obtained from all the owners, all the necessary information about the study was provided to all the participants before obtaining their consent.

Chapter IV

Biochar Supplementation of Recycled Manure Solids: Impact on their characteristics and GHG Emissions during storage

---

Ana José Pires, Catarina Esteves, Ricardo Bexiga, Manuela Oliveira, David Fangueiro – Submited for publibication in Agronomy

Authors' contributions:

Ana Pires: Set up of the experiment, performed the experiments, data analysis, drafting, critical revision, and final approval of the manuscript.

Catarina Esteves: helped to setup and to perform the experiments, data analysis and manuscript revision.

Ricardo Bexiga: contributed to the revision the manuscripts and supervision.

Manuela Oliveira: contributed to the revision the manuscripts and supervision throughout.

David Fangueiro: Set-up of the experiment; critical revision of the manuscripts, final manuscript approval and supervision throughout.

Abstract:
Recycled manure solids (RMS) are increasingly adopted in dairy farming for their economic advantages and their role in improving nutrient recycling and waste management; however, concerns regarding greenhouse gas (GHG) emissions during storage persist. This study assessed the effects of biochar supplementation at 2.5% (2.5B) and 10% (10B), compared to untreated RMS (C-) and acidified RMS (C+), on GHG emissions, measured continuously and intermittently, and RMS characteristics during one-month storage period. Results showed that addiction of biochar increased micronutrient and heavy metal concentration (with the exception of molybdenum (Mo)), as well as potassium (K) and magnesium (Mg) content by the end of the 1-month period. However, it decreased sodium (Na), phosphorus (P) and total organic carbon (TOC) content. Among treatments, 10B consistently demonstrated the greatest reductions in emissions with a decrease, relative to C- treatment, of 32% for carbon dioxide (CO_2), 47% for nitrous oxide (N_2O) and 32% regarding the global warming potential (GWP). Cumulative methane (CH_4) emissions did not show significant differences. Continuous monitoring captured transient emission peaks, highlighting the importance of high-resolution assessments. Despite the emissions generated during biochar production, its application in RMS bedding systems offsets these environmental costs by mitigating GHG emissions and increasing nutrient content. These findings highlight the potential of biochar application as a sustainable manure management strategy. Future research should focus on long-term field trials and optimizing biochar production to maximize environmental benefits and cost-effectiveness in dairy farming.

Keywords: Biochar, Recycled Manure Solids, Greenhouse Gas Emissions, Fertilizing Value, Dairy farming sustainability

1. Introduction

Reducing greenhouse gas (GHG) emissions is a key priority in the European Union's (EU) climate strategy, with the agriculture and livestock sector being a significant contributor (The European Green Deal, 2019). The European Green Deal and the "Farm to Fork" strategy aim to promote sustainable agricultural practices, including reducing emissions in the dairy sector (A Farm to Fork Strategy for a Fair, Healthy and Environmentally-Friendly Food System, 2020).

Dairy farming is a significant contributor to global GHG emissions, with manure management being the second largest source of these emissions at farm scale, just after enteric methane (CH_4) production. Manure is responsible for approximately 7% of both agricultural CH_4 and nitrous oxide (N_2O) emissions (Silva & Cabrera, 2024; US-EPA, 2006). Both gases play a crucial role in climate change and air quality deterioration, making environmentally-friendly

manure management practices essential in reducing the environmental footprint of dairy farming (Fangueiro et al., 2018).

Several practices for manure management, such as anaerobic digestion (AD), composting, solid-liquid (S/L) separation or the application of chemical additives, have potential to mitigate GHG emissions. S/L separation leads to the production of two new materials: a liquid fraction and a solid fraction. This technique has been shown to reduce GHG emissions by 46%, relative to stored raw manure (Yan et al., 2024). It is also a cost-effective strategy for managing manure, and when combined with other technologies, like AD or composting, S/L separation, can significantly enhance the overall efficiency of manure management systems and indirectly reduce CH_4 emissions (Yan et al., 2024). The solid fraction can also be used as bedding materials for dairy cows, known as recycled manure solids (RMS), but little is known about the impact of such practice on GHG emissions during its storage.

Acidification is another manure management recognized as effective for reducing emissions during manure storage and treatment Yan et al., 2024). The lower pH environment allows an efficient decrease of N_2O during acidified slurry storage (Prado et al., 2020) as well as during acidified RMS storage (Regueiro et al., 2016). However, while effective, the use of acidifiers such as sulfuric acid (H_2SO_4) can pose environmental challenges, including increased hydrogen sulfide (H_2S) emissions and potential over-fertilization with sulfur (S) during field application (Fangueiro et al., 2015; Regueiro et al., 2016).

Recently, manure amendment with biochar has emerged as a promising solution for improving manure management sustainability on dairy farms (Fangueiro et al., 2017; Ma et al., 2024; Sapkota et al., 2024). Produced through the controlled pyrolysis of organic materials, biochar features a high surface area and porous structure that enables it to adsorb various substances, including pollutants, while also potentially reducing GHG emissions (Fangueiro et al., 2017; Ma et al., 2024; Sapkota et al., 2024). Biochar has also proved to be efficient to enhance the fertilizing value of manure and mitigate environmental risks, hence RMS amendment with biochar could also be a successful strategy to mitigate GHG emissions during storage and increase its fertilizing value (Bello et al., 2020; Du et al., 2023; Sapkota et al., 2024).

Our study aimed to investigate the impact of biochar addition to RMS on GHG emissions during a one-month storage experiment and its characteristics. This approach was compared to RMS acidification, which served as a control (alongside untreated RMS). A small-scale, experiment was conducted under controlled conditions to evaluate the impact of acidification and varying biochar concentrations, by assessing RMS' chemical characteristics and GHG emissions at various time points.

2. Materials and Methods

2.1 RMS treatment

RMS was collected from a commercial dairy farm near Lisbon, Portugal, and mechanically separated prior to the experiment. The treatments used in this experiment consisted of:

1) RMS without supplementation, serving as the negative control (C-);
2) RMS supplemented with 10% sulfuric acid (H_2SO_4) serving as the positive control, (C+): acidification was performed by the addition of 20ml of 10% H_2SO_4 to one kg of RMS to reach a final pH of 5. The 10% H_2SO_4 was obtained by diluting the concentrated sulfuric acid (H_2SO_4, 98% w/w) in distilled water.
3) RMS with 2.5% biochar (2.5B);
4) RMS with 10% biochar (10B).

The percentages of biochar addiction reflect weight-by-weight (w/w) ratios, so for 1 kg of mixture it was added 25 g and 100 g of biochar in the 2.5B and 10B treatments, respectively. Each treatment was thoroughly mixed to ensure an even distribution of the biochar or H_2SO_4 throughout the RMS.

2.2 Small scale Experiment

A 30-day incubation experiment was performed using the five materials previously described to assess the impact of RMS treatment on GHG emissions during storage and final fertilizing value of RMS.

For the 4 treatments, 1 kg of the treated RMS was placed into hermetically sealed glass containers (5 L). The jars were equipped with inlets and outlets to allow air sampling and continuous gas monitoring. These containers were then stored in a controlled environment where temperature (ranging between 15ºC and 19ºC) and humidity (ranging between 57% and 82%) were maintained at levels representative of typical storage conditions in dairy farms. Each treatment group was replicated three times.

2.3 Chemical Analysis of RMS and Biochar

Samples were collected from each treatment group at two time points: Day 0 (immediately after treatment application) and Day 30 (end of incubation). Additionally, samples from the biochar used were analyzed in triplicate. The samples were then analyzed following the methods described by Prado et al. (Prado et al., 2020). Briefly, dry matter (DM) content was determined by drying fresh samples in a drying oven at 105°C for 24 hours (Heraeus Function Line, Thermo Fisher Scientific, USA). The organic matter (OM) content was determined by combusting the dried samples in a furnace at 550°C for 3 hours (B180, Naberttherm, Germany). Subsequently, the total organic carbon (TOC) was calculated by dividing OM

content by a conversion factor of 1.8, considering that 58% of OM content consists of organic carbon (C). The pH and electrical conductivity (EC) were determined by preparing a suspension with deionized water in a 1:10 ratio (m/v). Following this, pH was measured using an Orion 3 Star pH meter (Thermo Fisher Scientific, USA), while EC was measured using an Orion Star A212 EC meter (Thermo Fisher Scientific, USA).

The total nitrogen (Ntotal) and ammoniacal nitrogen (N-NH_4^+) concentrations were determined using the Kjeldahl method (Horneck and Miller, 1998). Ntotal was measured by digesting the samples followed by distillation and titration, while NH_4^+-N was measured using only the distillation and titration steps.

The concentrations of total phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), sodium (Na), iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), boron (B), molybdenum (Mo), chromium (Cr) and nickel (Ni), cadmium (Cd) and lead (Pb), were measured following a digestion procedure. Approximately 0.2–0.3 g of oven-dried sample were digested in a mixture of 9 mL of nitric acid and 3 mL of hydrogen peroxide at 100°C using a block digestion system (Digipress MS, SCP Science, Canada). The concentrations of these elements were determined using an inductively coupled plasma optical emission spectrometer (ICP-OES, iCAP 7000 Series, Thermo Fisher Scientific, USA).

2.4 Gas Emissions Monitoring

Throughout the 30-day incubation period, CH_4, N_2O, and CO_2 were continuously monitored. The monitoring system utilized a Stand-Alone Multipoint Sampler (Innova 1409, LumaSense Technologies A/S, Denmark) connected to a Photoacoustic Field Gas-Monitor (Innova 1412i, LumaSense Technologies A/S, Denmark). This setup enabled simultaneous monitoring of gas samples from every container. The sampler was set up according to the procedures described in the system's instruction manual (LumaSense Technologies A/S, 2017), and gas measurements were conducted using pre-calibrated filters specific for each gas: CO_2 (UA 0982), CH_4 (UA 0969), and N_2O (UA 0985) with the respective detection limits of 5.1 ppm, 0.4 ppm and 0.03 ppm. The photoacoustic gas monitor was also equipped with an optical filter for water vapor (filter type SB0527) and prior to measurement was configurated to compensate for water interference and cross-interference.

Additionally, measurements of CH_4, N_2O, CO_2 emissions were taken manually (hereon after mentioned as intermittent sampling), without being attached to the sampler, twice a day in the initial 5 days, and then with less frequency as the experiment progressed.

2.5 Calculation and Data Analysis

Gas fluxes from both the continous and intermittent sampling were calculated using the following equation adapted from Fangueiro et al. (Fangueiro et al., 2017).

$$Gas\ flux(mg\ Gas/kg/day) = \frac{\frac{G \times F}{1000} \times 1440}{RMS's\ Weight}$$

Where:

- G is the gas concentration in mg/m³ from the Innova system.
- F is the airflow rate, set at 2.2 L/min.
- The value 1440 is the number of minutes in a day, used to convert the reading to daily emissions.
- RMS's weight is the weight of the RMS sample in kilograms.

Global warming potential (GWP) of each treatment was calculated by converting the emissions of CO_2, CH_4 and N_2O into their carbon dioxide equivalents (CO_2-eq), using the conversion factors of 1, 27.2 and 273, respectively (Intergovernmental Panel on Climate Change (IPCC), 2023), which are the 100-year global warming potential values.

The cumulative emissions were then calculated by averaging the gas flux between two consecutive sampling points and multiplying by the time interval between those points.

The dataset was carefully inspected for potential outliers that could compromise the reliability of the analysis. Outliers were identified through statistical methods, ensuring that any anomalies were substantiated by contextual evidence from the experimental setup and potential setbacks (such as airflow interruptions).

To evaluate the effect of biochar supplementation on cumulative gas emissions (CO_2, N_2O, CH_4) and GWP, a comprehensive statistical analysis was performed using R (version 4.0.3, R Core Team, 2023). The data were first inspected for normality using the Shapiro-Wilk test.

For metrics that did not meet the assumptions of normality and homogeneity of variances, Kruskal-Wallis tests were conducted as the non-parametric alternative. Where significant effects were found, pairwise comparisons were conducted using Dunn's test with Bonferroni correction.

For metrics that met the assumptions of normality and homogeneity of variances, an Analysis of Variance (ANOVA) was applied using the aov() function in R. The model included the treatment as a fixed effect. Where significant effects were found ($p \leq 0.05$), pairwise comparisons were conducted using the LSD method.

3. Results

3.1 Impact of Biochar Supplementation on RMS Characteristics

At Day 0, it was already visible some differences: acidification of RMS (C+ treatment) significantly decreased the pH and increased EC, while biochar supplementation increased DM content, especially the 10B treatment (Table 15). At this day, higher Ntotal content was

observed in the negative control (C-) and higher S content was observed in the C+ treatment (Table 16). Additionally, biochar supplementation increased Fe, Mn, B and Pb content, compared with C- (Table 17).

By the end of the experiment, a similar trend of lower pH and higher EC persisted in the C+ treatment. However, at this point, DM was higher in the C- treatment and lower in the 10B treatment.; and TOC content was higher and lower in the C- and 10B treatments, respectively. Furthermore, higher $N\text{-}NH_4^+$ content was observed in the C- and C+ treatments, while Ntotal content showed no significant differences between treatments. Higher nutrient content was observed in the 10B treatment, namely K, C and Mg content. In contrast, the 10B treatment, along with 2.5B, showed the lowest content of P and S, whereas the C+ treatment showed the highest content of these two nutrients, followed by C-. The C- treatment showed the highest Na content, while 10B showed the lowest.

Regarding the other elements presented at Table 17, the highest concentration was consistently found in the 10B treatment, followed by 2.5B and lowest in the C+ and C- treatments. The only exception was Mo content, which was lowest with the 10B treatment.

Biochar was also analysed, and results showed the relatively high pH and EC values and high DM and TOC content. It was also observed that biochar was relatively poor in $N\text{-}NH_4^+$ and Ntotal content, but high nutrient content, namely K, Ca, Mg, Fe, Mn, B and heavy metals (Cr, Ni and Pb).

Table 15 – Mean values (n=3) ± standard error of pH, electrical conductivity (EC), dry matter (DM) and total organic carbon (TOC) per treatment at Day 0 and Day 30. 10B - RMS with 10% biochar supplementation; 2.5B - RMS with 2.5% biochar supplementation; C- - negative control (RMS without supplementation); C+ - positive control (acidified RMS). Superscript letters denote statistical differences ($p<0.05$).

	Treatment	pH	EC	DM	TOC
			mS cm^{-1}	g kg^{-1} (DM)	
Day 0	**C-**	$9.30^{a} \pm 0.05$	$0.466^{c} \pm 0.01$	$287.60^{b} \pm 14.20$	150.80 ± 7.39
	C+	$3.81^{b} \pm 1.52$	$3.452^{a} \pm 1.81$	$272.80^{b} \pm 4.79$	142.00 ± 2.20
	2.5B	$9.15^{a} \pm 0.08$	$0.488^{bc} \pm 0.00$	$294.40^{ab} \pm 24.49$	148.00 ± 7.11
	10B	$9.17^{a} \pm 0.05$	$0.526^{b} \pm 0.00$	$322.50^{a} \pm 16.59$	150.00 ± 3.92
Day 30	**C-**	$8.22^{a} \pm 0.31$	$0.143^{c} \pm 0.05$	$444.70^{a} \pm 1.67$	$227.90^{a} \pm 0.55$
	C+	$5.06^{b} \pm 0.16$	$0.770^{a} \pm 0.12$	$314.30^{c} \pm 0.05$	$160.00^{c} \pm 0.63$
	2.5B	$8.66^{a} \pm 0.21$	$0.610^{ab} \pm 0.05$	$383.50^{b} \pm 20.20$	$212.40^{b} \pm 11.16$
	10B	$8.58^{a} \pm 0.13$	$0.497^{bc} \pm 0.03$	$280.40^{d} \pm 8.41$	$131.00^{d} \pm 6.97$
Biochar		9.69 ± 0.03	0.518 ± 0.02	852.57 ± 180.47	245.10 ± 69.77

Table 16 - Mean values (n=3) ± standard error of main macronutrients per treatments at Day 0 and at Day 30. DM – dry matter; 10B - RMS with 10% biochar supplementation; 2.5B - RMS with 2.5% biochar supplementation; C- - negative control (RMS without supplementation); C+ - positive control (acidified RMS). Superscript letters denote statistical differences ($p<0.05$).

	Treatment	$N\text{-}NH_4^+$	Ntotal	Na	K	Ca	Mg	P	S
		g kg^{-1} (DM)							
Day 0	C-	1.64 ± 0.03	20.67^{a} ± 3.56	0.96^{a} ± 0.00	5.53^{b} ± 0.01	10.25^{a} ± 0.20	3.58^{a} ± 0.12	2.51^{a} ± 0.02	3.39^{b} ± 0.07
	C+	1.79 ± 0.12	18.95^{ab} ± 0.88	0.85^{b} ± 0.01	4.82^{c} ± 0.06	8.80^{c} ± 0.12	2.76^{b} ± 0.58	2.23^{b} ± 0.03	13.83^{a} ± 0.57
	2.5B	1.93 ± 0.62	16.41^{bc} ± 1.33	0.85^{b} ± 0.02	5.14^{c} ± 0.04	9.39^{b} ± 0.09	3.28^{b} ± 0.00	2.18^{b} ± 0.05	2.89^{c} ± 0.00
	10B	0.97 ± 0.11	13.76^{c} ± 1.11	0.70^{c} ± 0.00	5.73^{a} ± 0.16	9.70^{b} ± 0.29	3.55^{a} ± 0.21	1.69^{c} ± 0.06	2.15^{c} ± 0.02
Day 30	C-	0.82^{a} ± 0.17	21.20 ± 1.43	1.12^{a} ± 0.07	6.57^{b} ± 0.00	11.34^{b} ± 0.14	4.00^{c} ± 0.00	2.67^{a} ± 0.03	3.85^{b} ± 0.04
	C+	0.85^{a} ± 0.08	21.60 ± 4.09	1.02^{b} ± 0.01	5.79^{c} ± 0.05	10.35^{c} ± 0.90	3.64^{d} ± 0.04	2.60^{b} ± 0.05	11.75^{a} ± 0.70
	2.5B	0.72^{ab} ± 0.09	20.00 ± 0.68	1.01^{b} ± 0.02	6.72^{b} ± 0.15	11.51^{b} ± 0.01	4.23^{b} ± 0.10	2.35^{c} ± 0.03	3.30^{c} ± 0.10
	10B	0.67^{b} ± 0.04	18.20 ± 1.50	0.84^{c} ± 0.02	6.84^{a} ± 0.08	12.70^{a} ± 0.12	4.68^{a} ± 0.06	2.01^{d} ± 0.04	2.41^{d} ± 0.03
Biochar		0.18 ± 0.10	2.28 ± 0.04	0.52 ± 0.05	7.60 ± 1.09	16.06 ± 1.39	5.95 ± 0.68	1.22 ± 0.36	0.91 ± 0.30

Table 17 – Mean values (n=3) ± standard error of main micronutrients and heavy metals per treatment at Day 0 and Day 30. DM – dry matter; 10B - RMS with 10% biochar supplementation; 2.5B - RMS with 2.5% biochar supplementation; C- - negative control (RMS without supplementation); C+ - positive control (acidified RMS). Superscript letters denote statistical differences ($p<0.05$).

	Treatment	Fe	Cu	Zn	Mn	B	Mo	Cr	Ni	Cd	Pb
		mg kg^{-1} (DM)									
Day 0	C-	1386^{c} ± 75.61	25 ± 1.62	139 ± 0.96	106^{c} ± 0.42	39^{b} ± 2.14	2.06^{a} ± 0.07	4.66 ± 0.74	3.19 ± 0.40	0.25 ± 0.02	0.15^{b} ± 0.16
	C+	1131^{d} ± 13.96	23 ± 0.26	129 ± 0.10	95^{d} ± 0.82	33^{c} ± 0.74	1.82^{a} ± 0.02	4.02 ± 0.01	2.57 ± 0.30	0.24 ± 0.03	0.36^{b} ± 0.03
	2.5B	1699^{b} ± 213.06	22 ± 0.06	128 ± 2.12	148^{b} ± 5.24	39^{b} ± 2.53	1.51^{b} ± 0.21	5.00 ± 0.36	2.82 ± 0.88	0.26 ± 0.02	1.22^{b} ± 0.05
	10B	7111^{a} ± 176.59	22 ± 1.79	127 ± 11.26	449^{a} ± 100.80	75^{a} ± 14.51	1.12^{c} ± 0.08	15.29 ± 0.75	3.20 ± 0.46	0.61 ± 0.05	2.69^{a} ± 0.30
Day 30	C-	1409^{c} ± 68.85	28^{b} ± 0.07	153 ± 6.60	124^{c} ± 2.95	44^{c} ± 0.59	2.29^{a} ± 0.20	4.54^{c} ± 0.21	2.61^{b} ± 0.14	0.25^{c} ± 0.01	0.16^{c} ± 0.26
	C+	1259^{d} ± 18.46	24^{c} ± 0.56	137 ± 10.57	121^{c} ± 6.59	35^{d} ± 4.61	2.13^{a} ± 0.10	4.29^{c} ± 0.10	2.58^{b} ± 0.15	0.24^{c} ± 0.01	0.02^{c} ± 0.01
	2.5B	2784^{b} ± 100.02	27^{b} ± 0.13	158 ± 0.78	271^{b} ± 14.10	53^{b} ± 0.31	1.93^{a} ± 0.02	9.71^{b} ± 0.26	2.97^{b} ± 0.28	0.39^{b} ± 0.03	1.28^{b} ± 0.22
	10B	6331^{a} ± 17.10	29^{a} ± 0.85	152 ± 10.36	573^{a} ± 5.92	84^{a} ± 0.02	1.50^{b} ± 0.22	15.39^{a} ± 0.43	3.78^{a} ± 0.11	0.74^{a} ± 0.03	2.90^{a} ± 0.49
Biochar		14691.56 ± 2294.31	27.75 ± 3.54	149.97 ± 47.65	1181.33 ± 95.46	121.03 ± 51.61	1.95 ± 2.53	27.28 ± 9.87	38.15 ± 57.88	1.02 ± 0.28	7.66 ± 2.59

3.2 GHG Emissions and GWP

3.2.1 Continuous Sampling

The temporal trends in CO_2 emissions (Figure 12 - a) showed distinct patterns across treatments, with several peaks observed during the monitoring period. On Day 0, the biochar-amended treatments recorded the highest emissions, with 2.5B and 10B registering 17.04 and 15.91 mg CO_2 d^{-1} kg^{-1}, respectively. The controls, C+ and C-, exhibited slightly lower emissions, peaking at 14.37 and 15.54 mg CO_2 d^{-1} kg^{-1}, respectively. Up until Day 3, emissions were in the following descending order: 2.5 < C+ < C- < 10B. Afterwards, C- started emitting more CO_2 than C+, but 2.5B and 10B still maintained high and low emissions, respectively. There were several other peaks between day 7 and 14, and at the end of this period, the emissions in the 2.5B treatment were very similar to those of the controls. By the end of the monitoring period, emissions were similar between all treatments, ranging between 4.5 and 5.5 mg CO_2 d^{-1} kg^{-1}, with lower emissions in the biochar-amended treatments.

N_2O emissions (Figure 12 - b) showed some variation throughout the monitoring period, albeit small, varying from 1.75 to 3.17 mg N_2O d^{-1} kg^{-1} across all treatments. Significant peaks were however observed: at Day 4 all treatments peaked, but emission values were highest in the C+, 2.5B and 10B treatments, reaching 2.73, 2.75 and 3.17 mg N_2O d^{-1} kg^{-1}, respectively. There were five more relevant peaks: at day 7, highest with the 10B treatment; at day 8, highest with the C+ treatment; at day 9, highest with the 2.5B treatment; at day 21, highest with the 10B treatment; and finally, at day 23, highest with the C- treatment.

Regarding CH_4 emissions (Figure 12 - c), several peaks were observed throughout the experiment. The most noticeable were the peaks on Days 4, 7, 11 and 24, which were highest for the 10B treatment (reaching 7.13 µg CH_4 d^{-1} kg^{-1} on Day 4), followed by 2.5B, C+ and finally C-. Between day 11 and 12, there were relevant emissions in all treatments, but besides this occurrence, CH_4 emissions were virtually non existent.

Cumulative emissions over the entire continuous monitoring period (Table 18) showed higher and lower CO_2 emissions in the 2.5B and 10B treatments, respectivelly. It was also observed higher CH_4 emissions in the 10B treatment, and lowest in the controls, though cumulative emissions were relatively low. This led to higher GWP in the 2.5B treatment, and lowest in the 10B. The controls showed intermediate GWP values. N_2O emissions did not significantly differ between treatments.

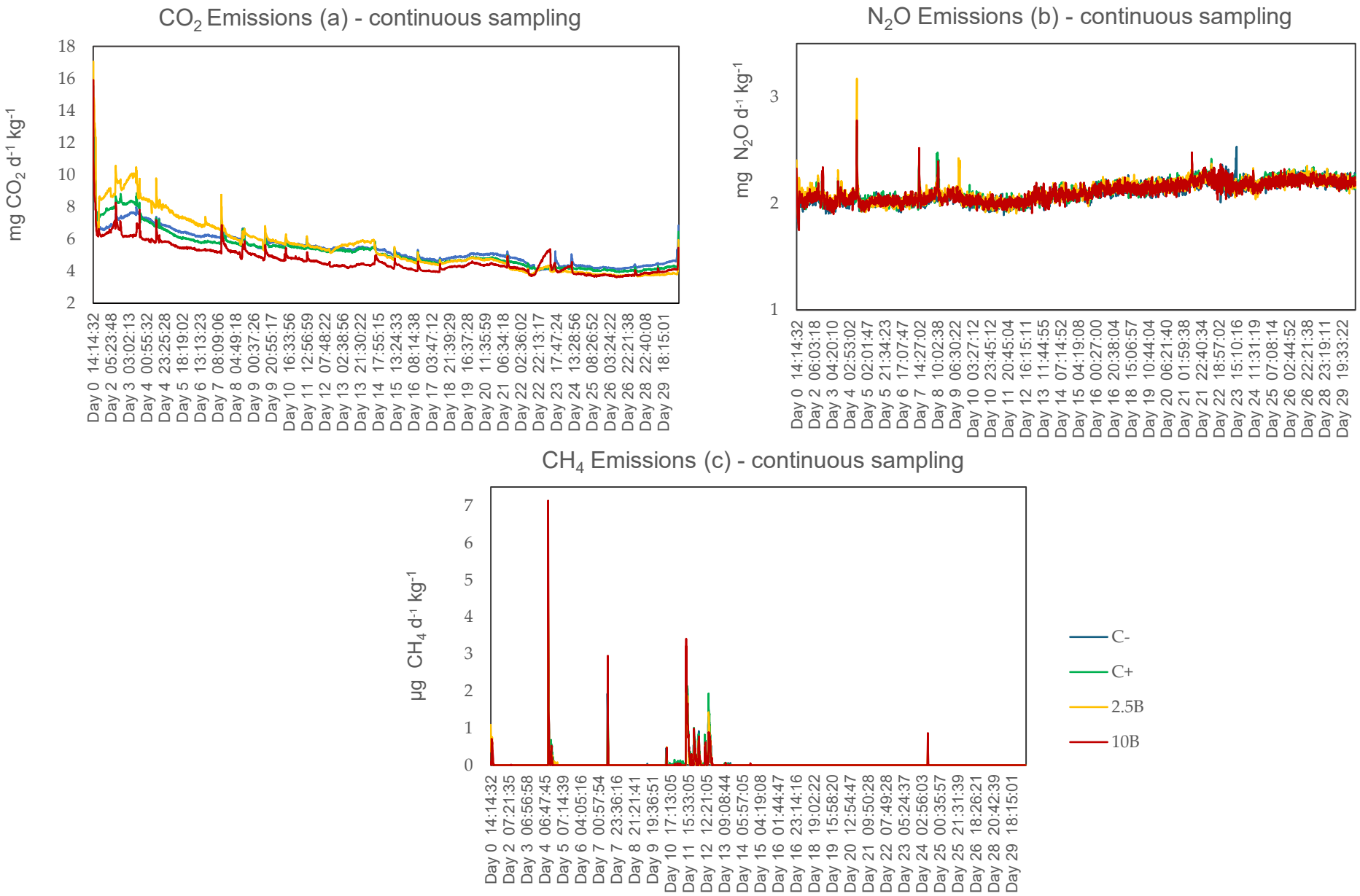


Figure 12 - Mean values (n=3) for CO_2 (a), N_2O (b) and CH_4 (c) emissions across different treatments during the experimental period from the continuous monitoring system. 10B - RMS with 10% biochar supplementation; 2.5B - RMS with 2.5% biochar supplementation; C- - negative control (RMS without supplementation); C+ - positive control (acidified RMS). To be noted that the CO_2 and N_2O y-axis begin on 2 and 1, respectively.

Table 18 - Mean values (n=3) ± standard error for the cumulative GHG emissions and GWP for each treatment at the end of the continous monitoring period. 10B - RMS with 10% biochar supplementation; 2.5B - RMS with 2.5% biochar supplementation; C- - negative control (RMS without supplementation); C+ - positive control (acidified RMS). Superscript letters denote statistical differences ($p<0.05$).

Treatment	CO_2	N_2O	CH_4	GWP
	g kg^{-1}	mg kg^{-1}		g CO_2-eq kg^{-1}
C-	249.61^{ab} ± 14.85	96.73 ± 0.52	1.39^{b} ± 0.24	275.77^{ab} ± 14.99
C+	243.91^{ab} ± 13.42	97.64 ± 0.51	1.60^{b} ± 0.02	270.37^{ab} ± 13.55
2.5 B	260.77^{a} ± 15.60	97.59 ± 0.32	1.66^{ab} ± 0.04	287.23^{a} ± 15.56
10 B	217.40^{b} ± 4.19	97.02 ± 0.33	1.73^{a} ± 0.04	243.61^{b} ± 4.16

3.2.2 Intermittent Sampling

Regarding the emissions that were measured intermittently, the temporal trends were similar between CO_2, N_2O and CH_4: an initial high peak, followed by a sharp decline and subsequent stabilization with little variation. Exceptionally, CH_4 emissions followed the initial peak were non-existent.

The initial peak in CO_2 emissions was (Figure 13 - a) highest for the C- and C+ treatments, reaching 100.49 and 80.94 mg CO_2 d^{-1} kg^{-1}, respectively, while in the 2.5B and 10B this peak was smaller, reaching 44.25 and 32.02 mg CO_2 d^{-1} kg^{-1}, respectively. In terms of N_2O emissions (Figure 13 - b), the initial peak was highest with C+, followed by C-, 2.5B and 10B treatments, reaching 7.42, 5.33, 2.65 and 1.62 mg N_2O d^{-1} kg^{-1}, respectively. While the initial CH_4 peak was highest in the 10B and 2.5B treatments (Figure 13 – c), reaching 1.53 and 1.44 mg CH_4 d^{-1} kg^{-1}, respectively.

Cumulative emissions from the intermittent monitoring system (Table 19) showed higher CO_2 emissions in the C- treatment and lower in the 10B treatment, higher N_2O emissions in the C-, C+ and 2.5B treatments, and lower in the 10B treatment. This resulted in higher GWP in the C- treatment, and lowest in the 10B treatment. No significant differences were observed in terms of cumulative CH_4 emissions.

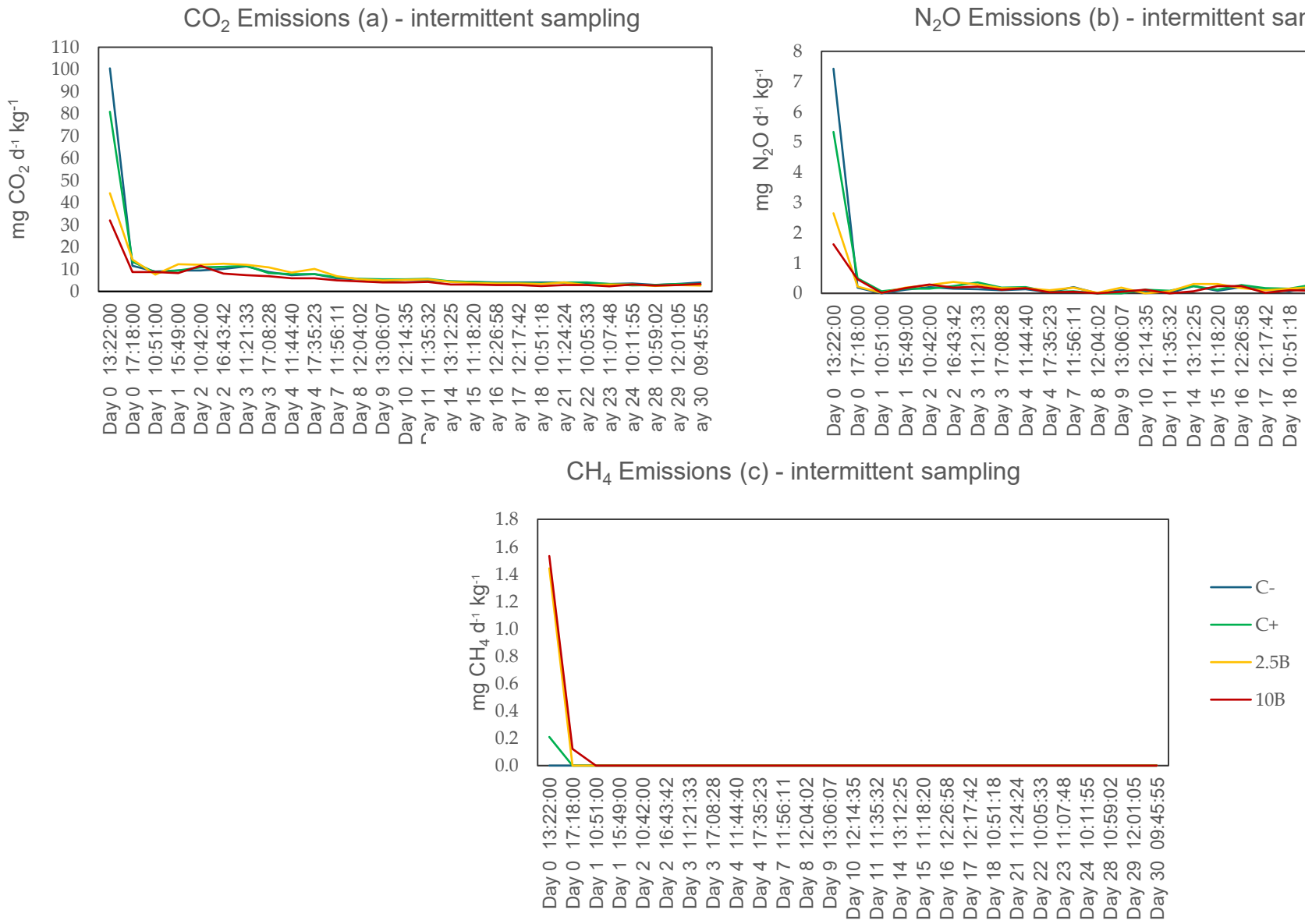


Figure 13 - Mean values (n=3) for CO_2 (a), N_2O (b) and CH_4 (c) emissions across different treatments during the experimental period from the intermittent monitoring system. 10B - RMS with 10% biochar supplementation; 2.5B - RMS with 2.5% biochar supplementation; C- - negative control (RMS without supplementation); C+ - positive control (acidified RMS).

Table 19 - - Mean values (n=3) ± standard error for the cumulative GHG emissions and global warming potential (GWP) for each treatment at the end of the intermittent monitoring period. 10B - RMS with 10% biochar supplementation; 2.5B - RMS with 2.5% biochar supplementation; C- - negative control (RMS without supplementation); C+ - positive control (acidified RMS). Superscript letters denote statistical differences ($p<0.05$).

Treatment	CO_2	N_2O	CH_4	GWP
	g kg^{-1}	mg kg^{-1}		g CO_2-eq kg^{-1}
C-	$415.60^{a} \pm 4.27$	$13.53^{a} \pm 2.49$	0.00 ± 0.00	$419.29^{a} \pm 3.62$
C+	$402.68^{ab} \pm 15.51$	$13.39^{a} \pm 2.04$	0.23 ± 0.39	$406.34^{ab} \pm 15.93$
2.5 B	$372.75^{bc} \pm 15.95$	$10.94^{a} \pm 1.07$	1.56 ± 2.70	$375.78^{bc} \pm 15.89$
10 B	$282.17^{c} \pm 21.70$	$7.19^{b} \pm 1.25$	1.84 ± 2.93	$284.18^{c} \pm 21.87$

4. Discussion

4.1 Impact of Biochar Supplementation on RMS Characteristics

Acidification significantly reduced the pH of RMS, even after 30 days of storage, while increasing EC values and S content. This was to be expected, considering the effectiveness of H_2SO_4 in acidifying liquid and solid manure (Sørensen & Eriksen, 2009). It has also been reported in previous studies, the positive impact of acidification on EC values [9] and S content (Eriksen et al., 2008). By the end of the experiment, C- showed lower DM and TOC content than C-. Previous studies have found that acidification retained higher OM content by inhibiting microbial activity (Sørensen & Eriksen, 2009). However, this was found for pig slurry. This indicated that acidification has different impacts depending on the material being acidied. More studies using RMS should be conducted to understand the underlying mechanisms of acidification on microbial activity.

In contrast, supplementation with biochar did not alter the pH of RMS. It did, however, increased EC values, only significantly in the 2.5B treatment in comparison with C-. This suggested a higher concentration of dissolved ions, potentially due to increased solubility of nutrients, or the release of mineral components from biochar. A similar outcome was observed in a soil experiment, where biochar supplementation increased soil EC values (Burrell et al., 2016)

DM plays a fundamental role in RMS stability, influencing microbial activity, moisture balance, and nutrient availability. By the end of the storage period, DM content was lower in the biochar-amended treatments, in comparison with C-. Similarly, TOC levels were lower with biochar supplementation. These results may indicate that mineralization of organic C might have occurred. Which aligns with previous studies, where biochar may have promoted microbial activity, potentially accelerating OM mineralization (Busch et al., 2024). Vieira Firmino et al. (Vieira Firmino et al., 2024) reported that biochar initially stabilizes DM but can later facilitate shifts in moisture availability, depending on microbial activity and aeration, impacting both DM and TOC.

Regarding N content, immediately after application of biochar, Ntotal and N-NH_4^+ content seemed lower in the biochar-amended treatments, indicating that adsorption of N might have taken place in the initial moments after trial setup or immobilization by soil's microrganims (Brunn et al., 2011). By the end of the experiment, higher N-NH_4^+ content was found in the C- and C+ treatments, compared with the 10B treatment, indicating fewer N losses.

Biochar supplementation also increased nutrient content as well as heavy metal content, likely due to an already rich composition of these elements (such as K, Ca, Mg, Fe, Cu, Mn, BCr, Cd and Pb) in the biochar composition. It could also be due to adsorption of these elements

in the biochar particles, due to its high surface area and porosity, and its surface charges (Hossain et al., 2020) Regarding specifically the heavy metal content, although biochar supplementation resulted in an increase, the content levels were not nearly near the legal limits for sludge and soil according to Portuguese regulations (CBPA, 2018)

4.2 GHG Emissions and GWP

4.2.1 Continuous Sampling

The initial CO_2 peak, especially high in the biochar-amended treatments, might be due to the rapid mineralization of labile organic C and the stimulation of microbial respiration, a trend observed in studies showing that biochar enhances microbial activity through improved aeration and moisture regulation (Shrestha et al., 2023). The subsequent decline in CO_2 emissions aligns with biochar's role in stabilizing organic C (Shrestha et al., 2023). This aligns with the previous results of lower DM and TOC content in the biochar-amended treatments, further sustaining the hypothesis that biochar supplementation increased microbial activity and OM decomposition. However, the effect of biochar dose seemed to impact CO_2 emissions, considering that 2.5B resulted in significantly higher emissions that 10B. A review from Shrestha et al. (2023) found that biochar has varying impacts on CO_2 emissions, from increasing to decreasing emissions; and in case of reductions, these were not proportional to biochar supplementation dose (Shrestha et al., 2023). However, as many of the studies with biochar, these results are obtained from soil studies. The same can be hypothetized here, that different biochar doses have different impact on CO_2 emissions from RMS: lower doses increased microbial activity, while higher doses stabilized organic matter decomposition. Although, further experiments are necessary to confirm such results using RMS.

Regarding N_2O emissions, emissions peaks were observed from all treatments. The initial peak was higher than the other peaks observed later in the experiment, and might be explained by higher C and N availability (Yang et al., 2020). Despite these peaks, cumulative N2O emissions did not significantly differ between treatments.

This pattern suggests that biochar's capacity to adsorb NH_4^+ slows its conversion to nitrate and subsequent N_2O emission (Liu et al., 2021). Studies have reported that biochar amendments can reduce N_2O emissions by modifying N availability and microbial community composition, delaying nitrification while still supporting microbial activity (Shrestha et al., 2023; Yang et al., 2020). Biochar's moisture regulation and NH_4^+ adsorption may have influenced these delayed peaks by altering substrate availability for nitrifying and denitrifying microbes (Shrestha et al., 2023; Yang et al., 2020).

CH_4 emissions exhibited sporadic peaks resulting from transient anaerobic conditions favoring methanogenesis. Additionally, condensation inside storage containers may have contributed

to localized anaerobic conditions, affecting methane release dynamics. Nevertheless, CH_4 emissions were residual, amounting to cumulative emissions lower than 2 mg kg^{-1} during the entire 30-day experiment. By the end of the experiment, the highest dose of biochar significantly increased cumulative CH_4 emissions. Liu et al. (Liu et al., 2021) similarly reported elevated CH_4 emissions from biochar-amended liquid pig manure, attributing this effect to biochar's interaction with slurry dynamics. Although their study used liquid manure, the findings align with those of the present experiment.

The calculated GWP was lower in the 10B treatment compared with 2.5B, however, neither of the treatments differed from C-. The overall reduction in GWP in the 10B treatment suggests that biochar can be an effective mitigation strategy when applied at sufficient rates to stabilize OM and modify microbial pathways responsible for GHG production (Busch et al., 2024). However, the elevated emissions and GWP observed in the 2.5B treatment emphasize the need for further investigation into how lower biochar dosages influence microbial activity and C mineralization dynamics during RMS storage.

4.2.2 Intermittent Sampling

Like the continuous monitoring results, CO_2 emissions exhibited a pronounced peak on Day 0 across all treatments. However, in contrast with the continuous monitoring, the highest values were recorded in the C- and C+ treatments. This rapid release corresponds to microbial decomposition of labile organic C, commonly observed in manure decomposition (Shrestha et al., 2023). Biochar-amended RMS displayed lower peaks, suggesting biochar's role in stabilizing C (M. Zhang et al., 2024). Following this peak, emissions declined across all treatments.

Cumulative CO_2 emissions were significantly lower in the 10B treatment compared to the controls, while the 2.5B treatment did not differ from C+. This trend mirrors the continuous monitoring findings, further reinforcing biochar's role in reducing CO_2 emissions through C stabilization (Shrestha et al., 2023), especially at higher dosages. The slightly higher cumulative emissions in 2.5B than in 10B suggest that lower biochar dosages may enhance microbial respiration by improving aeration and accessibility to organic substrates (M. Zhang et al., 2024), as previously observed.

N_2O emissions also peaked on Day 0, with lower values in biochar-amended treatments, particularly 10B. This reduction is likely due to biochar's NH_4^+ adsorption capacity, limiting substrate availability for nitrification (Busch et al., 2024). Like CO_2, N_2O emissions stabilized quickly across treatments after the initial peak, with no further marked fluctuations. Cumulative N_2O emissions were significantly lower in the 10B treatment compared to the controls and 2.5B treatment. The reductions in N_2O emissions in 10B align with previous findings that biochar can mitigate emissions by reducing N availability for microbial transformations (M.

Zhang et al., 2024). The lack of effect in 2.5B suggests that lower biochar doses may not provide sufficient adsorption capacity to alter N cycling processes (Vieira Firmino et al., 2024). CH_4 emissions exhibited sharp peaks on Day 0, particularly high in 10B and 2.5B, followed by a rapid decline to zero. These patterns suggest transient anaerobic conditions in the early decomposition stages, followed by the establishment of aerobic conditions (Shrestha et al., 2023). Cumulative CH_4 emissions were very low across all treatments, with no significant differences between treatments, indicating that while biochar may promote initial peaks, it does not necessarily alter total CH_4 emissions under the aerobic conditions of this study (M. Liu et al., 2021).

The cumulative GWP showed significantly lower values in the 10B and 2.5B treatments compared to C-, which was unlike the results from the continuous monitoring. Witth the intermittent sampling tecnhique, the reduction in GWP compared with C- was primarily driven by decreases in CO_2 and N_2O emissions, showcasing biochar's potential as a mitigation strategy for GHG emissions (Shrestha et al., 2023).

These results confirm that biochar supplementation, particularly at 10%, effectively reduced CO_2 and N_2O emissions and GWP during RMS storage. While the limited effect on CH_4 highlighted the need for further studies to explore biochar's influence on methane dynamics under varying environmental conditions (Liu et al., 2021).

4.2.3 Sampling Methods Comparison

Continuous monitoring, with its higher frequency, captured transient fluctuations such as those observed in CO_2 emissions during Days 2–4, 7–11 and 15-17. These fluctuations were likely linked to changes in microbial respiration and oxygen availability, which were effectively captured in real-time. In contrast, intermittent sampling, with its longer intervals, provided a smoother emission profile that underestimated these rapid variations. The differences in cumulative CO_2 emissions between methods suggest that intermittent sampling slightly overestimated emissions by integrating data over longer periods, potentially amplifying values by smoothing temporary reductions in microbial activity (Liu et al., 2021).

For N_2O emissions, continuous monitoring captured short-lived but intense peaks, which were not detected in intermittent sampling. The peaks observed between Days 2–4 and Days 7–11 highlight the episodic nature of nitrification and denitrification processes, which are highly sensitive to oxygen availability and microbial activity. Similar findings were reported by Shrestha et al. (Shrestha et al., 2023), who emphasized that high-frequency monitoring is critical for accurately assessing N_2O emission variability in biochar-amended manure systems. The discrepancy between cumulative N_2O emissions recorded by each method underscores the limitations of intermittent sampling in capturing transient N fluxes. While cumulative N_2O emissions were higher under continuous monitoring, the intermittent method underestimated

total emissions by missing these critical peaks, reinforcing the importance of real-time data collection for gases with high temporal variability.

For CH_4, continuous monitoring detected sporadic short-lived peaks, whereas the intermittent method recorded a more stable emission pattern. The differences between methods were less pronounced for CH_4 than for CO_2 and N_2O, likely due to the predominantly aerobic conditions limiting methanogenesis. Consequently, cumulative CH_4 emissions showed no considerable differences between monitoring approaches, confirming that under well-aerated conditions, variations in temporal resolution have minimal influence on total CH_4 emissions (Liu et al., 2021).

These findings highlight the importance of selecting monitoring methods that align with the emission characteristics of each gas. Continuous monitoring provides detailed temporal resolution, capturing transient peaks and variations that are particularly critical for gases like N_2O. Intermittent sampling, on the other hand, smooths out short-term fluctuations, offering a practical approach for capturing long-term trends and overall cumulative impacts.

4.3 Biochar Carbon Footprint: Balancing Production Emissions and RMS GHG Reduction

Biochar production through pyrolysis generates GHG emissions, particularly CO_2 and CH_4, with estimates suggesting that 10-20% of feedstock carbon is lost as CO_2 depending on the pyrolysis temperature and system efficiency (Handiso et al., 2024; Meyer et al., 2011). Despite these emissions, biochar's long-term C sequestration potential and its capacity to mitigate GHGs during manure management systems can offset these initial production costs (Li et al., 2024; Wu et al., 2023; Patwa et al., 2022).

In this study, the application of biochar at 10% (10B) to RMS reduced cumulative (intermitent sampling) CO_2 and N_2O emissions by approximately 32% and 47%, respectively, translating into an overall GWP reduction of 32%, compared with untreated RMS. These findings align with literature highlighting biochar's ability to stabilize organic C and suppress N cycling processes responsible for N_2O production (Li et al., 2024; Patwa et al., 2022). The reduction in GWP achieved here suggests that biochar can potentially offset production-related emissions, particularly when considering its long-term stability and sequestration potential.

Overall, these results underscore biochar's potential to act as a C-negative strategy in manure management by mitigating GHG emissions while balancing the trade-offs associated with its production phase. Optimizing production processes to further reduce emissions could enhance biochar's environmental benefits and sustainability.

Chapter V

General Discussion, conclusion and future perspectives

1.General Discussion

Antimicrobial resistance is recognized as one of the most critical challenges to public health, not only threatening the efficacy of modern antibiotics but also impacting animal health, environmental safety, and food safety (Baquero, 2021; Lee, 2019; Riley, 2005). The rapid emergence and spread of antimicrobial resistant pathogens are largely driven by the misuse and overuse of antibiotics in human and veterinary medicine, alongside the application of antimicrobials in agriculture (Iskandar et al., 2022; Munita & Arias, 2016). As bacteria develop mechanisms to resist antimicrobial treatments, infections that were once easily treatable become increasingly difficult to manage, resulting in higher morbidity, mortality, and healthcare costs (Browne et al., 2020; Sharma et al., 2017; Vos et al., 2020).

In veterinary medicine, AMR presents challenges for disease control, as it complicates the management of infectious diseases, including in livestock, increasing the risk of outbreaks and impacting animal welfare and productivity. The spread of resistant bacteria within livestock production, including in dairy farms, is particularly concerning as it may facilitate the transmission of resistance genes to commensal and pathogenic bacteria within these systems (Sharma et al., 2017; Tiseo et al., 2020). The World Organization for Animal Health (WOAH / OIE), together with the World Health Organization, advocates for responsible antibiotic stewardship across veterinary and agricultural practices, encouraging measures to reduce antibiotic use, promote alternative treatments, and enhance infection control (World Health Organization, 2015).

Dairy farming may play a significant role in the development and spread of AMR, and its environmental impacts. Antibiotics are frequently used in dairy farms to treat infections, particularly mastitis, a prevalent disease that significantly affects milk production and animal welfare (Tiseo et al., 2020; Van Boeckel et al., 2017). While these antibiotics are essential for maintaining individual animal health, their use in large-scale dairy production systems creates an environment that may select for resistant bacteria, which can persist in animal manure and spread through various pathways (de Greeff et al., 2022; Oliver et al., 2020). When administered, antibiotics not only impact the targeted pathogens but also exert selective pressure on commensal and opportunistic bacteria, promoting the evolution of ARG within these microbial communities (Oliver et al., 2011).

Cattle manure may act as a vehicle for resistant bacteria, as well as for pathogenic bacteria that pose risks to both animal and human health (Heinonen-Tanski et al., 2006; Oliver et al., 2020). Within dairy operations, bacteria such as *Escherichia coli* and *Enterococcus* spp. are of particular concern due to their prevalence in the gastrointestinal tracts of cows and their high potential for resistance. These bacteria are not only indicators of fecal contamination but also serve as reservoirs of ARG, which can spread through horizontal gene transfer within bacterial communities (Barlow et al., 2017; Ekore et al., 2022; Jacobs et al., 2019; Ji et al.,

2023; Kolenda et al., 2015). When manure is applied to agricultural fields as fertilizer or soil amendment, resistant bacteria can enter the soil microbiota, where they may share resistance genes with other soil bacteria, including those capable of infecting humans and other animals (Buta-Hubeny et al., 2022; Oliver et al., 2020; Zalewska et al., 2021).

The environmental impacts of manure extend beyond AMR-related concerns, as manure management in dairy farming is associated with several ecological challenges. The decomposition of manure can contribute to the release of greenhouse gases (GHGs), including methane (CH_4) and nitrous oxide (N_2O), both of which have a potent global warming potential and play substantial roles in climate change (Fangueiro et al., 2018; da Silva & Cabrera, 2024; US-EPA, 2006).

Therefore, both AMR dissemination concerns and environmental impacts underscore the need for improved manure management practices within dairy farming, in order to reduce AMR transmission risks while also addressing broader ecological concerns (Pires et al., 2024).

To address these interconnected health and environmental issues, various manure treatment strategies have been developed. However, existing manure treatment methods, while beneficial in certain aspects, have limitations in effectively managing all these risks (Keenum et al., 2021; Yan et al., 2024).

Solid-liquid separation (S/L) effectively divides animal slurry (liquid manure) into a nutrient-dense liquid fraction and a solid fraction, enhancing nutrient recovery and repurposing the solid fraction as bedding or compost. However, resistant bacteria can persist in the separated fractions, especially in the solid bedding material, which may contribute for the reintroduction of these bacteria into animal housing areas, thus posing recurring health risks (Fangueiro et al., 2021; Guerreiro et al., 2024; Pires et al., 2025).

Subsequently, Anaerobic digestion (AD) can be used to convert the organic matter present in the solids fraction to biogas, thereby reducing methane emissions and generating renewable energy. However, the process requires significant infrastructure investment and does not fully eliminate pathogens, leaving secondary waste products that may still harbour resistant bacteria (Yan et al., 2024). It is still to refer that anaerobic digestion of slurry may happen during slurry storage due to croast formation or even when storage facilities are covered. In such case, anaerobic digestion might ensure parcial or total hygienization of the slurry even if the biogas produced is not recovered.

Composting is another common treatment applied to cow solid manure that may offer advantages such as improving soil fertility, enhancing microbial activity, and contributing to soil structure. However, its application in large-scale operations can be constrained by several challenges, including the need for prolonged processing time, substantial labor requirements, and the potential for greenhouse gas emissions. Additionally, achieving effective pathogen reduction often depends on maintaining optimal conditions, which can be difficult to ensure in

practical settings (Keenum et al., 2021; Yan et al., 2024).

Acidification is another available approach, which involves lowering the pH of manure to inhibit microbial activity and consequently significantly reduce ammonia emissions. While effective, acidification presents its own challenges, as the use of acidifiers such as sulfuric acid has to be performed only by trained staff and may lead to undesirable environmental effects, as the release of harmful sulphur compounds (Fangueiro et al., 2015). Moreover, acidification may not be suitable for all agricultural contexts, particularly those where there are concerns about soil acidification (Fangueiro et al., 2015; Yan et al., 2024).

Biochar, a carbon-rich material produced from the pyrolysis of organic biomass, has gained attention regarding its potential to address AMR, bacterial load, and environmental impacts in manure management (Zhang et al., 2014; X. Zhang et al., 2023). The unique properties of biochar, its high surface area, porosity, and adsorption capacity, allow it to capture a range of contaminants, including pathogens and antibiotic residues, thereby reducing bacterial load and minimizing the spread of AMR genes (Ma et al., 2024).

Biochar interacts with manure microbiota by adsorbing and immobilizing nutrients, heavy metals, and other compounds, potentially altering microbial activity and gene transfer rates. Research suggests that biochar's chemical and physical properties can suppress specific microbial populations, thus decreasing the prevalence of pathogenic bacteria and reducing the likelihood of resistance gene transfer (Ma et al., 2024; Perez-Mercado et al., 2019).

In addition to microbial control, biochar offers environmental benefits by mitigating greenhouse gas emissions from manure storage. Studies indicate that biochar can adsorb ammonia and decrease methane and nitrous oxide emissions, contributing to improved air quality and reduced carbon footprint in dairy farming (Ma et al., 2024; Sapkota et al., 2024). However, the overall carbon footprint reduction attributed to biochar use must be critically evaluated, as its production through pyrolysis involves the combustion of organic feedstocks, potentially contributing to GHG emissions. Thus, while biochar supplementation may mitigate emissions locally, its net environmental benefit depends on the balance between its production emissions and the emissions reductions achieved in manure management(Meyer et al., 2011; J. Zhang et al., 2024). Its ability to stabilize nutrients further enhances the agronomic value of treated manure as a fertilizer, promoting sustainable soil enrichment practices that align with the goals of climate-resilient agriculture (Bello et al., 2020; Du et al., 2023; Sapkota et al., 2024).

The research presented in this thesis builds on the potential of biochar as an alternative to mitigate the limitations presented by conventional treatments, investigating the effects of biochar supplementation in RMS intended for use as cow bedding.

The work presented in Chapter 2 investigated the AMR and virulence profiles of *Escherichia coli* and *Enterococcus* species isolated from biochar-supplemented Recycled Manure Solids (RMS), providing insights into the potential of biochar to limit the spread of pathogenic and

antimicrobial-resistant bacteria. Understanding these dynamics is essential to evaluate the broader implications of biochar supplementation in RMS to be used as bedding material in dairy farming, particularly its role in reducing microbial risks. Although the study provided valuable data, the lack of statistical significance in many observed trends, combined with the absence of consistently favorable outcomes, underscores the limitations in the applicability and reliability of these findings.

The results from Chapter 2 explored the potential of biochar supplementation to modulate bacterial communities in RMS, particularly focusing on its ability to mitigate antimicrobial resistance (AMR) risks and the pathogenic potential of *E. coli* and *Enterococcus* spp.. Observed trends indicated that the supplementation of RMS with biochar was associated with a decrease in *E. coli* and *Enterococcus spp.* counts over time; likely influenced by aerobic digestion processes, however, these reductions were not statistically significant. These findings align with previous research highlighting the importance of oxygen availability in decreasing bacterial counts and pathogen loads in manure systems (Hutchison et al., 2005; Gurtler et al., 2018; Rapp et al., 2023). However, the magnitude of reductions, particularly for *E. coli*, was lower than those reported for biochar applications in other systems, such as poultry manure, where biochar exhibited stronger suppressive effects on bacterial loads (Mohammadi-Aragh et al., 2022).

It was also possible to identify several *Enterococcus* species, commonly linked to the normal gut microbiota of cattle, in the RMS supplemented samples. While *E. hirae* and *E. gallinarum* pose a lower risk, the detection of *E. faecium* and *E. faecalis* represents a concern, as these species are known carriers of AMR and virulence determinants (Ramos et al., 2020; Willems et al., 2023). Their persistence within RMS samples throughout time suggests that while biochar can mitigate some microbial risks associated with RMS, certain bacterial species may remain unaffected or adapt to the biochar-supplemented environment.

In addition to bacterial counts, the study extended its analysis to characterize AMR and virulence profiles of the isolates, offering critical insights into their pathogenic potential. Although biochar supplementation showed trends of reduced resistance rates to certain antibiotics, such as oxytetracycline, these changes were not statistically significant. These results align with existing research linking high excretion rates of oxytetracycline in dairy manure to a high selective pressure for resistant bacterial populations (Oliver et al., 2020; Richardson et al., 2018). While the absence of multidrug resistant (MDR) isolates is promising, the persistence of resistance to certain antibiotics, such as oxytetracycline and ampicillin, suggests that the interactions between RMS, biochar supplementation, bacterial communities, and antimicrobial agents are complex and require further investigation.

The analysis of the virulence potential of the recovered isolates revealed variable results, with *Enterococcus* isolates obtained from RMS supplemented with 2.5% biochar showing a

reduction in their ability to produce biofilm and express proteinase. Similarly, *E. coli* isolates obtained from RMS treated with 5% biochar exhibited reduced virulence, with hemolysin being the only virulence factor expressed by them. These results suggest that the physical and chemical properties of biochar, such as its porous structure and high surface area, may create localized environments within RMS. These microenvironments could influence how bacteria attach to surfaces and compete for resources, potentially affecting their expression of virulence traits.

Building on the targeted bacterial analysis presented in Chapter 2, the research presented in Chapter 3 provided a broader perspective on the microbial dynamics within biochar-supplemented RMS, emphasizing shifts in composition and diversity of bacterial communities across RMS samples subjected to different biochar supplementations. RMS are known to harbour a diverse microbial community, including potential mastitis-causing pathogens such as *E. coli*, *Streptococcus* spp., *Enterococcus* spp., and *Staphylococcus* spp. (Gurtler et al., 2018; Hutchison et al., 2005). The targeted analysis presented in Chapter 2 showed reductions in *E. coli* and *Enterococcus* spp. populations and their associated AMR profiles, although these changes were not statistically significant. This underscores the need for further investigation to fully evaluate biochar's potential to reduce pathogen prevalence and resistance in RMS. Chapter 3 revealed significant reductions in pathogenic bacteria, including Enterobacteriaceae, *Streptococcus* spp., and *Staphylococcus* spp., in biochar supplemented samples. Despite not promoting the complete eradication of bacteria, biochar supplementation demonstrated potential in reducing bacterial loads of RMS, particularly in humid conditions.

The findings from Chapter 3 highlight that RMS supplementation with biochar at concentrations of 5% yielded an average reduction of 59.50% in *Enterobacteriaceae*, streptococci, enterococci, and staphylococci in the samples of the assay performed in the humid season. This reduction aligns with the hypothesis that biochar's efficacy is influenced by environmental factors such as relative humidity and temperature. In the assay performed during the dry season, reductions of approximately 41% were observed in RMS samples supplemented with 2.5% and 5% biochar, indicating that lower concentrations may be cost-effective under less humid conditions.

The seasonal variations in microbial diversity further support the dynamic interaction between biochar and microbial communities. As reported in previous studies, biochar supplementation can reduce microbial diversity, likely due to its ability to selectively suppress certain bacterial populations (Ma et al. 2024). In this study, this effect was more pronounced during the humid season, where higher relative humidity may enhance biochar’s microbial modulation. While reduced diversity could be advantageous in lowering pathogenic populations, its ecological implications warrant further investigation.

In addition to reducing *Salmonella* spp. and pathogenic Gram-positive bacilli, biochar supplementation also decreased the abundance of thermoresistant streptococci and enterococci in RMS, which are known to affect milk quality and cheese production (Gagnon et al., 2020). These findings reinforce biochar's potential to mitigate risks associated with zoonotic and foodborne pathogens, addressing critical challenges in dairy farming and public health.

However, the work described in Chapter 3 also identified certain limitations of biochar supplementation. The increase in *Brucella* spp. levels in biochar-supplemented RMS highlights potential unintended consequences of biochar application. Previous studies have documented that charcoal-like substances can stimulate *Brucella* growth, emphasizing the need for routine monitoring and pathogen-specific mitigation strategies (Gorelick et al., 1951; Mena-Bueno et al., 2022). Similarly, an increase in *Pseudomonas* spp. during the humid season was also observed. While the reasons for these dynamics remain unclear, factors such as humidity, temperature, and biochar's physicochemical properties likely play a role.

The findings from Chapters 2 and 3 suggest that biochar supplementation may influence microbial dynamics in RMS, indicating a potential role in addressing microbial challenges in dairy farming. However, the lack of statistical significance in Chapter 2 highlights the need for further research to clarify these effects.

Finally, Chapter 4 expands the focus of the study, by investigating the environmental and chemical impacts of RMS supplementation with biochar. While promising trends were observed in GHG emissions and nutrients' stabilization, the results presented in this chapter also underscore the limitations and complexities of biochar applications under real-world conditions.

Biochar supplementation influenced the physicochemical properties of RMS, including pH, electrical conductivity (EC), dry matter (DM), ash content, and nitrogen dynamics. Unlike acidification, which significantly reduced RMS pH as expected (Fangueiro et al., 2018), biochar had no significant effect on pH stabilization. However, EC was higher in 2.5% biochar, possibly due to increased solubility of nutrients and the release of mineral components, a pattern previously observed in soil biochar studies (Burrell et al., 2016).

DM and Total organic carbon (TOC) content were lower in biochar-amended RMS, particularly in 10% biochar, suggesting increased microbial mineralization of organic matter (Vieira Firmino et al., 2024). The higher ash content in 10% biochar also indicates greater incorporation of biochar's mineral fraction, which could impact microbial community dynamics.

Regarding nitrogen, biochar adsorbed N-NH_4^+ initially, but by the end of storage, higher nitrogen retention was observed in 10% biochar compared to controls, potentially reducing volatilization losses (Busch et al., 2024).

In addition to these effects, biochar supplementation modified the nutrient profile of RMS. By the end of the storage period, biochar-treated RMS exhibited increased levels of micronutrients and heavy metals, with the exception of molybdenum (Mo). Additionally, supplementation enhanced potassium (K) and magnesium (Mg) availability, while simultaneously reducing sodium (Na), phosphorus (P), and total organic carbon (TOC) content. These findings highlight biochar's role in nutrient stabilization and its potential implications for the agronomic value of RMS. However, further investigation is necessary to fully understand its impact on nutrient cycling and soil fertility.

The observed shifts in physicochemical properties also align with microbial trends from Chapters 2 and 3, where biochar supplementation influenced microbial diversity and reduced pathogenic loads. In particular, the stabilization of organic matter and nitrogen in biochar-treated RMS may have contributed to microbial community alterations, with 5% biochar showing the most pronounced effects on microbial suppression. Meanwhile, the analysis from Chapter 4 showed that 2.5% biochar retained more bioavailable nutrients, which could have contributed to the microbial persistence observed in the previous chapters. This suggests that biochar dosage plays a key role in balancing nutrient retention and microbial suppression. Recent studies support this observation, showing that lower biochar application rates enhance soil organic carbon, total nitrogen, promoting microbial activity and nutrient accumulation. In contrast, excessive biochar application can disrupt the soil carbon-to-nitrogen ratio, leading to imbalances that inhibit microbial growth and nutrient uptake (Zeden et al., 2023).

Biochar supplementation significantly influenced GHG emissions, particularly CO_2 and N_2O, while CH_4 emissions remained unchanged. The initial CO_2 peak in biochar-amended RMS suggests enhanced microbial respiration and organic carbon mineralization, which aligns with findings that biochar can improve aeration and microbial activity (Sapkota et al., 2024). However, while some studies suggest that higher biochar doses provide more available carbon to microbes, increasing microbial respiration and CO_2 emissions (Barbosa et al., 2024), our results showed a different trend, where 10B resulted in lower CO_2 emissions than 2.5B. This indicates that, in RMS systems, higher biochar doses may promote organic matter stabilization rather than enhanced microbial respiration. Over time, CO_2 emissions declined, reinforcing biochar's role in carbon retention. 2.5B exhibited higher CO_2 emissions than 10B, suggesting that lower doses of biochar may initially enhance microbial respiration, while higher doses create conditions that limit rapid organic carbon mineralization.

N_2O emissions followed a similar trend, with significantly lower cumulative emissions in 10B. Biochar's ability to adsorb NH_4^+ and regulate nitrogen availability likely contributed to delayed nitrification and denitrification, reducing overall emissions (Shrestha et al., 2023). In contrast, CH_4 emissions were sporadic, suggesting transient anaerobic conditions rather than a consistent biochar effect.

Overall, the reduction in GHG emissions observed in RMS samples supplemented with 10% biochar highlights its potential to mitigate environmental impacts of RMS storage. Intermittent measurements revealed that the 10% biochar treatment significantly reduced CO_2 emissions by 32% and N_2O emissions by 47%, resulting in a 32% decrease in global warming potential (GWP) compared to the untreated RMS, demonstrating biochar's efficacy in stabilizing organic carbon and inhibiting some nitrogen cycling processes. These findings are consistent with the studies by Vieira Firmino et al. (2024) and Busch et al. (2024), which highlight biochar's role in mitigating GHG emissions.

The results from Chapters 2 to 4 highlight biochar's multifaceted effects on RMS management, providing key insights into its impact on nutrient stabilization, microbial suppression, and emissions mitigation.

The stabilization of organic matter and nitrogen in biochar-amended RMS correlates with the microbial shifts observed in Chapter 3, particularly the reduction in pathogenic bacteria. However, 2.5% biochar retained more bioavailable nutrients, potentially supporting microbial activity rather than suppressing it, as seen in Chapter 2, where microbial reductions were not statistically significant. These findings indicate that the relationship between biochar dosage and microbial suppression is complex, requiring further investigation into optimal dosages for maximizing both environmental and hygienic benefits.

The observed GHG emission reductions in 10% biochar align with the microbial community changes reported in Chapter 3. Studies have demonstrated that biochar amendments can reduce N_2O emissions by increasing nitrogen retention and delaying nitrification (Ma et al., 2024), aligning with the findings observed in Chapter 3, where the reduction in emissions likely influenced microbial alterations (Yan et al., 2024; Ma et al., 2024). However, biochar's dual role in microbial activity modulation and emission reduction necessitates further research to understand its long-term stability in different manure management systems.

Overall, 10% biochar treatment showed strong stabilization effects and reduced emissions but did not consistently enhance microbial suppression, suggesting that extensive stabilization may not always lead to improved microbial control. Conversely, 2.5% biochar retained more bioavailable nutrients, which could have contributed to microbial persistence but resulted in higher emissions.

The findings from Chapters 2 to 4 suggest that biochar-supplemented RMS has potential as a supplementary strategy for manure management. While reductions in microbial risks, antimicrobial resistance, and greenhouse gas emissions were observed, these outcomes were not consistently significant, limiting the robustness of the conclusions. These results highlight the need for a cautious interpretation of biochar's role and emphasize that it is not a standalone solution. Future research should focus on optimizing biochar application rates, evaluating long-term microbial stability, and assessing the broader sustainability implications

of biochar in manure treatment. Such efforts are necessary to fully understand its contribution to circular agriculture and environmental sustainability, aligning with global efforts to improve waste management in livestock production systems.

2.Study Limitations

Despite the promising results observed in this study, several limitations warrant consideration. These constraints highlight the complexities of implementing biochar as a sustainable manure management solution and emphasize areas for further exploration.

While trends in microbial reductions, AMR, virulence profiles, and GHG and NH3 emissions reduction were observed, many results lacked statistical significance. This limitation stems partly from the limited sample sizes, which may not fully capture the variability and complexities present in real-world farming environments. Larger-scale *in vitro* studies, mimicking the conditions observed in diverse agricultural systems, are necessary to further clarify our findings.

The 30-day experimental period applied provided valuable insights into the short-term effects of biochar supplementation; however, it was insufficient to assess long-term dynamics, particularly for nutrient stabilization, microbial community shifts, and GHG mitigation. Prolonged studies are needed to evaluate the sustained impact of biochar on RMS, soil health, and microbial ecosystems under field-scale conditions.

Seasonal variations in microbial diversity and biochar efficacy underscore the influence of environmental factors such as temperature and humidity in the effects of biochar supplementation. Future studies should explore the application of biochar under a broader range of environmental conditions, including extreme weather scenarios and diverse geographic settings.

The unexpected increases of certain bacterial groups in RMS samples supplemented with biochar, such as *Brucella* spp. and *Pseudomonas* spp., highlight potential unintended consequences of biochar supplementation. These findings underline the need for pathogen-specific risk assessments and mitigation strategies, particularly for species with significant zoonotic or agricultural implications.

The chemical and physical properties of biochar, which are influenced by feedstock type and pyrolysis conditions, can significantly impact its efficacy. While this study utilized pine-derived biochar, future research should investigate the effects of biochars produced from other feedstocks and at varying pyrolysis temperatures, aiming to identify the most effective formulations for RMS management.

Finally, the cost-effectiveness of biochar application remains a critical factor in its use as a RMS supplement. Production, transportation, and integration into existing farm management systems pose economic challenges, particularly for small and medium-sized farms.

Comprehensive cost-benefit analyses are needed to assess the scalability of biochar supplementation and to identify opportunities for financial incentives or policy support.

3.Conclusion

This study explored the potential of biochar supplementation in RMS as a strategy to mitigate microbial risks, antimicrobial resistance, and environmental impacts in dairy farming. Although some microbiological outcomes (e.g., *E. coli* and *Enterococcus spp.* loads) showed no statistically significant changes, relevant trends emerged—particularly the 5% biochar treatment, which consistently reduced pathogenic bacterial groups under humid conditions. However, the unexpected increase in *Brucella spp.* and *Pseudomonas spp.* highlights the complexity of microbial interactions in biochar-treated environments and underlines the need for further investigation.

From an environmental perspective, 10% biochar reduced CO_2 and N_2O emissions and contributed to organic matter and nitrogen stabilization. Nonetheless, CH_4 emissions remained unchanged, and nutrient dynamics varied across treatments, affecting the agronomic potential of RMS.

Overall, biochar supplementation shows promise as a complementary manure management strategy. Still, its effects are dose-dependent and influenced by environmental conditions. Further long-term and field-scale studies are required to validate its practical application and optimize dosage, supporting its integration into sustainable agriculture systems aligned with One Health goals.

4. Future Perspectives

This study represents an important step towards understanding the role of biochar supplementation in the sustainable management of RMS. Despite the lack of statistical significance in certain parameters, the findings highlight key areas for future research and potential applications.

One of the main objectives of this research was to determine the optimal biochar supplementation percentage for enhancing microbial safety, mitigating AMR, and reducing the environmental impacts of RMS. However, the results did not allow for the conclusive identification of an optimal concentration, as different supplementation levels demonstrated varying degrees of effectiveness depending on the microbial and environmental parameters analysed. Future studies should focus on refining experimental designs to better capture the nuances of biochar interactions within RMS. This includes exploring a broader range of biochar concentrations and incorporating more detailed sampling methodologies to assess

how supplementation levels influence microbial dynamics, AMR, virulence factors, and environmental outcomes.

One of the main priorities should be to expand research to field-scale studies, as the controlled experimental conditions used in this study may not fully capture the complexities of real-world dairy farming environments. Larger-scale in vitro studies mimicking dairy farm conditions would provide critical insights into how biochar performs under varying environmental conditions and management practices, helping to validate its efficacy and scalability.

Further research should also explore the long-term impacts of biochar on RMS microbial communities and its interactions with soil ecosystems when applied as fertilizer. Understanding these dynamics will provide a more comprehensive assessment of biochar's potential to reduce AMR risks and enhance nutrient cycling. Additionally, the potential for unintended microbial shifts, such as the increase in Brucella spp. and Pseudomonas spp. observed in this study, warrants closer investigation. Identifying the specific conditions under which these shifts may occur, and their potential implications for animal and human health, is crucial to ensuring biochar's safe application in farming systems.

Economic feasibility remains a critical area for future exploration. Studies should assess the cost-effectiveness of biochar production, transport, and application, as well as its integration with other manure management practices. Developing cost-efficient biochar production methods and exploring alternative feedstocks could significantly enhance its accessibility and adoption.

Finally, interdisciplinary collaboration between microbiologists, environmental scientists, and agricultural stakeholders is essential to fully leverage biochar's potential within the One Health framework. By addressing microbial safety, AMR mitigation, and environmental sustainability in tandem, biochar could play a pivotal role in advancing sustainable dairy farming practices.

These future directions aim to build on the findings of this research, addressing its limitations while exploring biochar's broader applications and potential as a transformative tool in agriculture and sustainable waste management.

References

A Farm to Fork Strategy for a Fair, Healthy and Environmentally-Friendly Food System (2020). https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52020DC0381

Abboud, Z., Galuppo, L., Tolone, M., Vitale, M., Puleio, R., Osman, M., Loria, G. R., & Hamze, M. (2021). Molecular Characterization of Antimicrobial Resistance and Virulence Genes of Bacterial Pathogens from Bovine and Caprine Mastitis in Northern Lebanon. *Microorganisms*, *9*(6), 1148. https://doi.org/10.3390/microorganisms9061148

Abdallah, A., Francoz, D., Berman, J., Dufour, S., & Buczinski, S. (2022). Association between transfer of passive immunity and health disorders in multisource commingled dairy calves raised for veal or other purposes: Systematic review and meta-analysis. *Journal of Dairy Science*, *105*(10), 8371–8386. https://doi.org/10.3168/jds.2021-21671

Ahmed, M. B., Zhou, J. L., Ngo, H. H., Guo, W., & Chen, M. (2016). Progress in the preparation and application of modified biochar for improved contaminant removal from water and wastewater. *Bioresource Technology*, *214*, 836–851. https://doi.org/10.1016/j.biortech.2016.05.057

Ajose, D. J., Oluwarinde, B. O., Abolarinwa, T. O., Fri, J., Montso, K. P., Fayemi, O. E., Aremu, A. O., & Ateba, C. N. (2022). Combating Bovine Mastitis in the Dairy Sector in an Era of Antimicrobial Resistance: Ethno-veterinary Medicinal Option as a Viable Alternative Approach. *Frontiers in Veterinary Science*, *9*, 800322. https://doi.org/10.3389/fvets.2022.800322

Akdeniz, N. (2019). A systematic review of biochar use in animal waste composting. *Waste Management*, *88*, 291–300. https://doi.org/10.1016/j.wasman.2019.03.054

Alanis, V. M., Zurakowski, M., Pawloski, D., Tomazi, T., Nydam, D. V., & Ospina, P. A. (2021). Description of the Characteristics of Five Bedding Materials and Association With Bulk Tank Milk Quality on Five New York Dairy Herds. *Frontiers in Veterinary Science*, *8*, 636833. https://doi.org/10.3389/fvets.2021.636833

Assoni, L., Milani, B., Carvalho, M. R., Nepomuceno, L. N., Waz, N. T., Guerra, M. E. S., Converso, T. R., & Darrieux, M. (2020). Resistance Mechanisms to Antimicrobial Peptides in Gram-Positive Bacteria. *Frontiers in Microbiology*, *11*. https://www.frontiersin.org/articles/10.3389/fmicb.2020.593215

Aust, V., Knappstein, K., Kunz, H.-J., Kaspar, H., Wallmann, J., & Kaske, M. (2013). Feeding untreated and pasteurized waste milk and bulk milk to calves: Effects on calf

performance, health status and antibiotic resistance of faecal bacteria. *Journal of Animal Physiology and Animal Nutrition*, *97*(6), 1091–1103. https://doi.org/10.1111/jpn.12019

Bairán, G., Rebollar-Pérez, G., Chávez-Bravo, E., & Torres, E. (2020). Treatment processes for microbial resistance mitigation: The technological contribution to tackle the problem of antibiotic resistance. *International Journal of Environmental Research and Public Health*, *17*(23), 1–20. https://doi.org/10.3390/ijerph17238866

Balemi, A., Gumi, B., Amenu, K., Girma, S., Gebru, M., Tekle, M., Ríus, A. A., D'Souza, D. H., Agga, G. E., & Kerro Dego, O. (2021). Prevalence of Mastitis and Antibiotic Resistance of Bacterial Isolates from CMT Positive Milk Samples Obtained from Dairy Cows, Camels, and Goats in Two Pastoral Districts in Southern Ethiopia. *Animals*, *11*(6), 1530. https://doi.org/10.3390/ani11061530

Baquero, F. (2021). Threats of antibiotic resistance: An obliged reappraisal. *International Microbiology*, *24*(4), 499–506. https://doi.org/10.1007/s10123-021-00184-y

Barlow, R. S., McMillan, K. E., Duffy, L. L., Fegan, N., Jordan, D., & Mellor, G. E. (2017). Antimicrobial resistance status of Enterococcus from Australian cattle populations at slaughter. *PLoS ONE*, *12*(5). https://doi.org/10.1371/journal.pone.0177728

Barman, P., Joshi, S., Sharma, S., Preet, S., Sharma, S., & Saini, A. (2023). Strategic Approaches to Improvise Peptide Drugs as Next Generation Therapeutics. *International Journal of Peptide Research and Therapeutics*, *29*(4), 61. https://doi.org/10.1007/s10989-023-10524-3

Bello, A., Deng, L., Sheng, S., Jiang, X., Yang, W., Meng, Q., Wu, X., Han, Y., Zhu, H., & Xu, X. (2020). Biochar reduces nutrient loss and improves microbial biomass of composted cattle manure and maize straw. *Biotechnology and Applied Biochemistry*, *67*(5), 799–811. https://doi.org/10.1002/bab.1862

Bennett, S., Fliss, I., Ben Said, L., Malouin, F., & Lacasse, P. (2022). Efficacy of bacteriocin-based formula for reducing staphylococci, streptococci, and total bacterial counts on teat skin of dairy cows. *Journal of Dairy Science*, *105*(5), 4498–4507. https://doi.org/10.3168/jds.2021-21381

Beyers, M., Duan, Y.-F., Jensen, L. S., & Bruun, S. (2022). Effect of natural and regulatory conditions on the environmental impacts of pig slurry acidification across different regions in Europe: A life cycle assessment. *Journal of Cleaner Production*, *368*, 133072. https://doi.org/10.1016/j.jclepro.2022.133072

Bogado Pascottini, O., LeBlanc, S. J., Gnemi, G., Leroy, J. L. M. R., & Opsomer, G. (2023). Genesis of clinical and subclinical endometritis in dairy cows. *Reproduction*, *166*(2), R15–R24. https://doi.org/10.1530/REP-22-0452

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K.,

Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2018). QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science (No. e27295v2). *PeerJ Inc.* https://doi.org/10.7287/peerj.preprints.27295v2

Bonardi, S., Cabassi, C. S., Fiaccadori, E., Cavirani, S., Parisi, A., Bacci, C., Lamperti, L., Rega, M., Conter, M., Marra, F., Crippa, C., Gambi, L., Spadini, C., Iannarelli, M., Paladini, C., Filippin, N., & Pasquali, F. (2023). Detection of carbapenemase- and ESBL-producing *Klebsiella pneumoniae* from bovine bulk milk and comparison with clinical human isolates in Italy. *International Journal of Food Microbiology*, *387*, 110049. https://doi.org/10.1016/j.ijfoodmicro.2022.110049

Bonhotal, J., Schwarz, M., & Staehr, E. (2010). Use of Dried Manure Solids as Bedding for Dairy Cows. *Cornell Waste Management Institute*. https://ecommons.cornell.edu/server/api/core/bitstreams/0dae2310-b7f4-48c3-8ccf-4a41bd89c55b/content

Bradley, A. J., Leach, K. A., Green, M. J., Gibbons, J., Ohnstad, I. C., Black, D. H., Payne, B., Prout, V. E., & Breen, J. E. (2018). The impact of dairy cows' bedding material and its microbial content on the quality and safety of milk—A cross sectional study of UK farms. *International Journal of Food Microbiology*, *269*, 36–45. https://doi.org/10.1016/j.ijfoodmicro.2017.12.022

Brown, K., Uwiera, R. R. E., Kalmokoff, M. L., Brooks, S. P. J., & Inglis, G. D. (2017). Antimicrobial growth promoter use in livestock: A requirement to understand their modes of action to develop effective alternatives. *International Journal of Antimicrobial Agents*, *49*(1), 12–24. https://doi.org/10.1016/j.ijantimicag.2016.08.006

Browne, K., Chakraborty, S., Chen, R., Willcox, M. D., Black, D. S., Walsh, W. R., & Kumar, N. (2020). A New Era of Antibiotics: The Clinical Potential of Antimicrobial Peptides. *International Journal of Molecular Sciences*, *21*(19), Article 19. https://doi.org/10.3390/ijms21197047

Bruun, E. W., Müller-Stöver, D., Ambus, P., & Hauggaard-Nielsen, H. (2011). Application of biochar to soil and N2O emissions: Potential effects of blending fast-pyrolysis biochar with anaerobically digested slurry. Euro-pean

Brunton, L. A., Duncan, D., Coldham, N. G., Snow, L. C., & Jones, J. R. (2012). A survey of antimicrobial usage on dairy farms and waste milk feeding practices in England and Wales. *Veterinary Record*, *171*(12), Article 12. https://doi.org/10.1136/vr.100924

Burrell, L. D., Zehetner, F., Rampazzo, N., Wimmer, B., & Soja, G. (2016). Long-term effects of biochar on soil physical properties. *Geoderma*, *282*, 96–102. https://doi.org/10.1016/j.geoderma.2016.07.019

Busch, F., Leal, O. dos A., Siebers, N., & Brüggemann, N. (2024). Biochar captures ammonium and nitrate in easily extractable and strongly retained form without stimulating greenhouse gas emissions during composting. *Journal of Environmental Quality*, *53*(6), 1099–1115. https://doi.org/10.1002/jeq2.20634

Buta-Hubeny, M., Korzeniewska, E., Hubeny, J., Zieliński, W., Rolbiecki, D., Harnisz, M., & Paukszto, Ł. (2022). Structure of the manure resistome and the associated mobilome for assessing the risk of antimicrobial resistance transmission to crops. *Science of The Total Environment*, *808*, 152144. https://doi.org/10.1016/j.scitotenv.2021.152144

Cao, L. T., Wu, J. Q., Xie, F., Hu, S. H., & Mo, Y. (2007). Efficacy of Nisin in Treatment of Clinical Mastitis in Lactating Dairy Cows. *Journal of Dairy Science*, *90*(8), 3980–3985. https://doi.org/10.3168/jds.2007-0153

Chaudhry, T. H., Aslam, B., Arshad, M. I., Alvi, R. F., Muzammil, S., Yasmeen, N., Aslam, M. A., Khurshid, M., Rasool, M. H., & Baloch, Z. (2020). Emergence of bla_{NDM-1} Harboring *Klebsiella pneumoniae* ST29 and ST11 in Veterinary Settings and Waste of Pakistan. *Infection and Drug Resistance*, *Volume 13*, 3033–3043. https://doi.org/10.2147/IDR.S248091

Chauhan, A. S., George, M. S., Chatterjee, P., Lindahl, J., Grace, D., & Kakkar, M. (2018). The social biography of antibiotic use in smallholder dairy farms in India. *Antimicrobial Resistance & Infection Control*, *7*(1), 60. https://doi.org/10.1186/s13756-018-0354-9

Chen, C., Guron, G. K., Pruden, A., Ponder, M., Du, P., & Xia, K. (2018). Antibiotics and Antibiotic Resistance Genes in Bulk and Rhizosphere Soils Subject to Manure Amendment and Vegetable Cultivation. *Journal of Environmental Quality*, *47*(6), Article 6. https://doi.org/10.2134/jeq2018.02.0078

Chen, C., Pankow, C. A., Oh, M., Heath, L. S., Zhang, L., Du, P., Xia, K., & Pruden, A. (2019). Effect of antibiotic use and composting on antibiotic resistance gene abundance and resistome risks of soils receiving manure-derived amendments. *Environment International*, *128*, 233–243. https://doi.org/10.1016/j.envint.2019.04.043

Chen, Q.-L., Fan, X.-T., Zhu, D., An, X.-L., Su, J.-Q., & Cui, L. (2018). Effect of biochar amendment on the alleviation of antibiotic resistance in soil and phyllosphere of Brassica chinensis L. *Soil Biology and Biochemistry*, *119*, 74–82. https://doi.org/10.1016/j.soilbio.2018.01.015

Cho, Y., & Yoon, K.-J. (2014). An overview of calf diarrhea—Infectious etiology, diagnosis, and intervention. *Journal of Veterinary Science*, *15*(1), Article 1. https://doi.org/10.4142/jvs.2014.15.1.1

Clinical and Laboratory Standards Institute. (2008). *CLSI M31 A3: Performance Standards for Antimicrobial Susceptibility Testing* (3th ed., Vol. 28). Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute. (2024a). *CLSI M100: Performance Standards for Antimicrobial Susceptibility Testing* (34th ed.). Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute. (2024b). *CLSI VET09: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals* (2nd ed., Vol. 38). Clinical and Laboratory Standards Institute.

Código de Boas Práticas Agrícolas (CBPA), (2018) *Portuguese Republic Diary* No. 25/2018, Series 2 of 2018-02-05 (2018), pp. 4123–4170.

Cole, K. J., & Hogan, J. S. (2016). Short communication: Environmental mastitis pathogen counts in freestalls bedded with composted and fresh recycled manure solids. *Journal of Dairy Science*, *99*(2), 1501–1505. https://doi.org/10.3168/jds.2015-10238

Creamer, A. E., Gao, B., & Zhang, M. (2014). Carbon dioxide capture using biochar produced from sugarcane bagasse and hickory wood. *Chemical Engineering Journal*, *249*, 174–179. https://doi.org/10.1016/j.cej.2014.03.105

Criscuolo, N. G., Pires, J., Zhao, C., & Van Boeckel, T. P. (2021). Resistancebank.org, an open-access repository for surveys of antimicrobial resistance in animals. *Scientific Data*, *8*(1), 189. https://doi.org/10.1038/s41597-021-00978-9

Cui, E., Wu, Y., Zuo, Y., & Chen, H. (2016). Effect of different biochars on antibiotic resistance genes and bacterial community during chicken manure composting. *Bioresource Technology*, *203*, 11–17. https://doi.org/10.1016/j.biortech.2015.12.030

Cunha, E., Valente, S., Nascimento, M., Pereira, M., Tavares, L., Dias, R., & Oliveira, M. (2021). Influence of the dental topical application of a nisin-biogel in the oral microbiome of dogs: A pilot study. *PeerJ*, *9*, e11626. https://doi.org/10.7717/peerj.11626

Dankar, I., Hassan, H., & Serhan, M. (2022). Knowledge, attitudes, and perceptions of dairy farmers regarding antibiotic use: Lessons from a developing country. *Journal of Dairy Science*, *105*(2), 1519–1532. https://doi.org/10.3168/jds.2021-20951

de Greeff, S., Kolwijck, E., Schoffelen, A., & Verduin, C. (2022). NethMap 2022. Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands in 2021 / MARAN 2022. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2021. *Rijksinstituut voor Volksgezondheid en Milieu* RIVM. https://doi.org/10.21945/RIVM-2022-0057

de Jong, E., Creytens, L., De Vliegher, S., McCubbin, K. D., Baptiste, M., Leung, A. A., Speksnijder, D., Dufour, S., Middleton, J. R., Ruegg, P. L., Lam, T. J. G. M., Kelton, D. F., McDougall, S., Godden, S. M., Lago, A., Rajala-Schultz, P. J., Orsel, K., Krömker, V., Kastelic, J. P., & Barkema, H. W. (2023). Selective treatment of nonsevere clinical mastitis does not adversely affect cure, somatic cell count, milk yield, recurrence, or culling: A systematic review

and meta-analysis. *Journal of Dairy Science*, *106*(2), 1267–1286. https://doi.org/10.3168/jds.2022-22271

De Liguoro, M., Cibin, V., Capolongo, F., Halling-Sørensen, B., & Montesissa, C. (2003). Use of oxytetracycline and tylosin in intensive calf farming: Evaluation of transfer to manure and soil. *Chemosphere*, *52*(1), 203–212. https://doi.org/10.1016/S0045-6535(03)00284-4

DeAngelis, M. M., Wang, D. G., & Hawkins, T. L. (1995). Solid-phase reversible immobilization for the isolation of PCR products. *Nucleic Acids Research*, *23*(22), 4742–4743. https://doi.org/10.1093/nar/23.22.4742

Doehring, C., & Sundrum, A. (2019). The informative value of an overview on antibiotic consumption, treatment efficacy and cost of clinical mastitis at farm level. *Preventive Veterinary Medicine*, *165*, 63–70. https://doi.org/10.1016/j.prevetmed.2019.02.004

Donnenberg, M. S. (2015). 220—Enterobacteriaceae. In J. E. Bennett, R. Dolin, & M. J. Blaser (Eds.), *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition)* (pp. 2503-2517.e5). W.B. Saunders. https://doi.org/10.1016/B978-1-4557-4801-3.00220-4

Doumith, M., Day, M. J., Hope, R., Wain, J., & Woodford, N. (2020). Improved Multiplex PCR Strategy for Rapid Assignment of the Four Major *Escherichia coli* Phylogenetic Groups. *Journal of Clinical Microbiology*, *50*(9), 3108–3110. https://doi.org/10.1128/jcm.01468-12

Down, P. M., Bradley, A. J., Breen, J. E., & Green, M. J. (2017). Factors affecting the cost-effectiveness of on-farm culture prior to the treatment of clinical mastitis in dairy cows. *Preventive Veterinary Medicine*, *145*, 91–99. https://doi.org/10.1016/j.prevetmed.2017.07.006

Du, L., Ahmad, S., Liu, L., Wang, L., & Tang, J. (2023). A review of antibiotics and antibiotic resistance genes (ARGs) adsorption by biochar and modified biochar in water. *Science of The Total Environment*, *858*, 159815. https://doi.org/10.1016/j.scitotenv.2022.159815

Duan, M., Zhang, Y., Zhou, B., Wang, Q., Gu, J., Liu, G., Qin, Z., & Li, Z. (2019). Changes in antibiotic resistance genes and mobile genetic elements during cattle manure composting after inoculation with *Bacillus subtilis*. *Bioresource Technology*, *292*. https://doi.org/10.1016/j.biortech.2019.122011

EFSA Panel on Biological Hazards (BIOHAZ), Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Fernández Escámez, P. S., Girones, R., Koutsoumanis, K., Lindqvist, R., Nørrung, B., Robertson, L., Ru, G., Sanaa, M., Simmons, M., Skandamis, P., Snary, E., Speybroeck, N., Kuile, B. T., … Herman, L. (2017). Risk for the development of Antimicrobial Resistance (AMR) due to feeding of calves with milk containing residues of antibiotics. *EFSA Journal*, *15*(1). https://doi.org/10.2903/j.efsa.2017.4665

Ekore, D. O., Onanga, R., Nguema, P. P. M., Lozano, C., & Kumulungui, B. S. (2022). The Antibiotics Used in Livestock and Their Impact on Resistance in *Enterococcus faecium* and *Enterococcus hirae* on Farms in Gabon. *Antibiotics*, *11*(2), 224. https://doi.org/10.3390/antibiotics11020224

Eldesoukey, I. E., Elmonir, W., Alouffi, A., Beleta, E. I. M., Kelany, M. A., Elnahriry, S. S., Alghonaim, M. I., alZeyadi, Z. A., & Elaadli, H. (2022). Multidrug-Resistant Enteropathogenic *Escherichia coli* Isolated from Diarrhoeic Calves, Milk, and Workers in Dairy Farms: A Potential Public Health Risk. *Antibiotics*, *11*(8), 999. https://doi.org/10.3390/antibiotics11080999

EMA. (2018, September 17). *Advice on impacts of using antimicrobials in animals* [Text]. European Medicines Agency.

Endimiani, A., Bertschy, I., & Perreten, V. (2012). *Escherichia coli* Producing CMY-2 β-Lactamase in Bovine Mastitis Milk. *Journal of Food Protection*, *75*(1), 137–138. https://doi.org/10.4315/0362-028X.JFP-11-320

Eriksen, J., Sørensen, P., & Elsgaard, L. (2008). The fate of sulfate in acidified pig slurry during storage and following application to cropped soil. *Journal of Environmental Quality*, *37*(1), 280–286. https://doi.org/10.2134/jeq2007.0317

European Food Safety Authority, European Centre for Disease Prevention and Control, & European Medicines Agency. (2021). *Antimicrobial consumption and resistance in bacteria from humans and animals: Third joint inter-agency report on integrated analysis of antimicrobial agent consumption and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals in the EU/EEA : JIACRA III 2016–2018*. Publications Office. https://data.europa.eu/doi/10.2900/056892

European Medicines Agency. (2022). *Sales of veterinary antimicrobial agents in 31 European countries in 2021: Trends from 2010 to 2021 : twelfth ESVAC report.* Publications Office. https://data.europa.eu/doi/10.2809/39517

Fangueiro, D., Alvarenga, P., & Fragoso, R. (2021). Horticulture and Orchards as New Markets for Manure Valorisation with Less Environmental Impacts. *Sustainability*, *13*(3), 1436. https://doi.org/10.3390/su13031436

Fangueiro, D., Hjorth, M., & Gioelli, F. (2015). Acidification of animal slurry– a review. *Journal of Environmental Management*, *149*, 46–56. https://doi.org/10.1016/j.jenvman.2014.10.001

Fangueiro, D., Lopes, C., Surgy, S., & Vasconcelos, E. (2012). Effect of the pig slurry separation techniques on the characteristics and potential availability of N to plants in the resulting liquid and solid fractions. *Biosystems Engineering*, *113*(2), 187–194. https://doi.org/10.1016/j.biosystemseng.2012.07.006

Fangueiro, D., Pereira, J. L. S., Fraga, I., Surgy, S., Vasconcelos, E., & Coutinho, J. (2018). Band application of acidified slurry as an alternative to slurry injection in a Mediterranean double cropping system: Agronomic effect and gaseous emissions. *Agriculture, Ecosystems & Environment*, *267*, 87–99. https://doi.org/10.1016/j.agee.2018.08.011

Fangueiro, D., Pereira, J. L. S., Macedo, S., Trindade, H., Vasconcelos, E., & Coutinho, J. (2017). Surface application of acidified cattle slurry compared to slurry injection: Impact on NH3, N2O, CO2 and CH4 emissions and crop uptake. *Geoderma*, *306*, 160–166. https://doi.org/10.1016/j.geoderma.2017.07.023

Farrell, S., Benson, T., McKernan, C., Regan, Á., Burrell, A. M. G., & Dean, M. (2023). Factors influencing dairy farmers' antibiotic use: An application of the COM-B model. *Journal of Dairy Science*, *106*(6), 4059–4071. https://doi.org/10.3168/jds.2022-22263

Fatima, F., Siddiqui, S., & Khan, W. A. (2021). Nanoparticles as Novel Emerging Therapeutic Antibacterial Agents in the Antibiotics Resistant Era. *Biological Trace Element Research*, *199*(7), 2552–2564. https://doi.org/10.1007/s12011-020-02394-3

Fávero, S., Portilho, F. V. R., Oliveira, A. C. R., Langoni, H., & Pantoja, J. C. F. (2015). Factors associated with mastitis epidemiologic indexes, animal hygiene, and bulk milk bacterial concentrations in dairy herds housed on compost bedding. *Livestock Science*, *181*, 220–230. https://doi.org/10.1016/j.livsci.2015.09.002

Fergestad, M. E., De Visscher, A., L'Abee-Lund, T., Tchamba, C. N., Mainil, J. G., Thiry, D., De Vliegher, S., & Wasteson, Y. (2021). Antimicrobial resistance and virulence characteristics in 3 collections of staphylococci from bovine milk samples. *Journal of Dairy Science*, *104*(9), 10250–10267. https://doi.org/10.3168/jds.2020-19988

Fernandes, M., Nóbrega Carneiro, C., Villada Rosales, A. M., Grilo, M., Ramiro, Y., Cunha, E., Nunes, T., Tavares, L., Sandi, J., & Oliveira, M. (2022). Antimicrobial resistance and virulence profiles of Enterobacterales isolated from two-finger and three-finger sloths (Choloepus hoffmanni and Bradypus variegatus) of Costa Rica. *PeerJ*, *10*, e12911. https://doi.org/10.7717/peerj.12911

Field, D., O' Connor, R., Cotter, P. D., Ross, R. P., & Hill, C. (2016). In Vitro Activities of Nisin and Nisin Derivatives Alone and In Combination with Antibiotics against Staphylococcus Biofilms. *Frontiers in Microbiology*, *7*. https://www.frontiersin.org/articles/10.3389/fmicb.2016.00508

Finore, I., Feola, A., Russo, L., Cattaneo, A., Di Donato, P., Nicolaus, B., Poli, A., & Romano, I. (2023). Thermophilic bacteria and their thermozymes in composting processes: A review. *Chemical and Biological Technologies in Agriculture*, *10*(1), 7. https://doi.org/10.1186/s40538-023-00381-z

Firth, C. L., Kremer, K., Werner, T., & Käsbohrer, A. (2021). The Effects of Feeding Waste Milk Containing Antimicrobial Residues on Dairy Calf Health. *Pathogens*, *10*(2), Article 2. https://doi.org/10.3390/pathogens10020112

Fisher, R. A., Gollan, B., & Helaine, S. (2017). Persistent bacterial infections and persister cells. *Nature Reviews Microbiology*, *15*(8), 453–464. https://doi.org/10.1038/nrmicro.2017.42

Font-Palma, C. (2019). Methods for the Treatment of Cattle Manure—A Review. *C*, *5*(2), 27. https://doi.org/10.3390/c5020027

Food and Agriculture Organization of the United Nations. (2021). *The FAO Action Plan on Antimicrobial Resistance 2021–2025*. FAO. https://doi.org/10.4060/cb5545en

Food and Drug Administration, US. (2022). *Summary Report On Antimicrobials Sold or Distributed for Use in Food-Producing Animals*. U.S. Government.

Freu, G., Garcia, B. L. N., Tomazi, T., Di Leo, G. S., Gheller, L. S., Bronzo, V., Moroni, P., & Dos Santos, M. V. (2023). Association between Mastitis Occurrence in Dairy Cows and Bedding Characteristics of Compost-Bedded Pack Barns. *Pathogens*, *12*(4), Article 4. https://doi.org/10.3390/pathogens12040583

Frey, Y., Rodriguez, J. P., Thomann, A., Schwendener, S., & Perreten, V. (2013). Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. *Journal of Dairy Science*, *96*(4), 2247–2257. https://doi.org/10.3168/jds.2012-6091

Gabibov, A. G., Dontsova, O. A., & Egorov, A. M. (2020). Overcoming Antibiotic Resistance in Microorganisms: Molecular Mechanisms. *Biochemistry (Moscow)*, *85*(11), 1289–1291. https://doi.org/10.1134/S0006297920110012

Gagnon, M., Hamelin, L., Fréchette, A., Dufour, S., & Roy, D. (2020). Effect of recycled manure solids as bedding on bulk tank milk and implications for cheese microbiological quality. *Journal of Dairy Science*, *103*(1), 128–140. https://doi.org/10.3168/jds.2019-16812

Galama, P. J., Ouweltjes, W., Endres, M. I., Sprecher, J. R., Leso, L., Kuipers, A., & Klopčič, M. (2020). Symposium review: Future of housing for dairy cattle. *Journal of Dairy Science*, *103*(6), 5759–5772. https://doi.org/10.3168/jds.2019-17214

Ganda, E. K., Bisinotto, R. S., Decter, D. H., & Bicalho, R. C. (2016). Evaluation of an On-Farm Culture System (Accumast) for Fast Identification of Milk Pathogens Associated with Clinical Mastitis in Dairy Cows. *PLOS ONE*, *11*(5), e0155314. https://doi.org/10.1371/journal.pone.0155314

Gandhi, N. N., Inzana, T. J., & Rajagopalan, P. (2023). Bovine Airway Models: Approaches for Investigating Bovine Respiratory Disease. *ACS Infectious Diseases*, *9*(6), 1168–1179. https://doi.org/10.1021/acsinfecdis.2c00618

Garzon, A., Habing, G., Lima, F., Silva-del-Rio, N., Samah, F., & Pereira, R. (2022). Defining clinical diagnosis and treatment of puerperal metritis in dairy cows: A scoping review. *Journal of Dairy Science*, *105*(4), 3440–3452. https://doi.org/10.3168/jds.2021-21203

Gaudino, M., Nagamine, B., Ducatez, M. F., & Meyer, G. (2022). Understanding the mechanisms of viral and bacterial coinfections in bovine respiratory disease: A comprehensive literature review of experimental evidence. *Veterinary Research*, *53*(1), 70. https://doi.org/10.1186/s13567-022-01086-1

Ghanbarpour, R., & Salehi, M. (2010). Determination of Adhesin Encoding Genes in *Escherichia coli* Isolates from Omphalitis of Chicks. *American Journal of Animal and Veterinary Sciences*, *5*(2), 91–96. https://doi.org/10.3844/ajavsp.2010.91.96

Ghatak, S., Singha, A., Sen, A., Guha, C., Ahuja, A., Bhattacharjee, U., Das, S., Pradhan, N. R., Puro, K., Jana, C., Dey, T. K., Prashantkumar, K. L., Das, A., Shakuntala, I., Biswas, U., & Jana, P. S. (2013). Detection of New Delhi Metallo-beta-Lactamase and Extended-Spectrum beta-Lactamase Genes in *Escherichia coli* Isolated from Mastitic Milk Samples. *Transboundary and Emerging Diseases*, *60*(5), 385–389. https://doi.org/10.1111/tbed.12119

Gomes, C., Ruiz-Roldán, L., Mateu, J., Ochoa, T. J., & Ruiz, J. (2019). Azithromycin resistance levels and mechanisms in *Escherichia coli*. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-42423-3

Gorelick, A. N., Mead, D. D., & Kelly, E. H. (1951). The growth of bacteria in a charcoal-cellophane system. *Journal of Bacteriology*, *61*(4), 507–513. https://doi.org/10.1128/jb.61.4.507-513.1951

Gosselin, V. B., Bodmer, M., Schüpbach-Regula, G., Steiner, A., & Meylan, M. (2022). Survey on the disposal of waste milk containing antimicrobial residues on Swiss dairy farms. *Journal of Dairy Science*, *105*(2), 1242–1254. https://doi.org/10.3168/jds.2021-20948

Guerreiro, J. F., Pires, A. J., Nunes, M., Esteves, A., Chambel, L., Pascoal, P., Pereira, M., Fangueiro, D., Tavares, L., Dias, R., Bexiga, R., & Oliveira, M. (2024). Biochar supplementation affects the microbiome of recycled manure solids for cow bedding: A metagenomic analysis. *Journal of Dairy Science*, S0022-0302(24)01420-6. https://doi.org/10.3168/jds.2024-25616

Gurmessa, B., Milanovic, V., Foppa Pedretti, E., Corti, G., Ashworth, A. J., Aquilanti, L., Ferrocino, I., Rita Corvaglia, M., & Cocco, S. (2021). Post-digestate composting shifts microbial composition and degrades antimicrobial resistance genes. *Bioresource Technology*, *340*, 125662. https://doi.org/10.1016/j.biortech.2021.125662

Gurtler, J. B., Doyle, M. P., Erickson, M. C., Jiang, X., Millner, P., & Sharma, M. (2018). Composting To Inactivate Foodborne Pathogens for Crop Soil Application: A Review.

Journal of Food Protection, *81*(11), 1821–1837. https://doi.org/10.4315/0362-028X.JFP-18-217

Gurunathan, S., Choi, Y.-J., & Kim, J.-H. (2018). Antibacterial Efficacy of Silver Nanoparticles on Endometritis Caused by *Prevotella melaninogenica* and *Arcanobacterum pyogenes* in Dairy Cattle. *International Journal of Molecular Sciences*, *19*(4), Article 4. https://doi.org/10.3390/ijms19041210

Haley, B. J., Kim, S. W., Cao, H., Karns, J. S., & Van Kessel, J. A. S. (2017). 265 Genomic and metagenomic analysis of antibiotic resistance in dairy animals. *Journal of Animal Science*, *95*(suppl_4), Article suppl_4. https://doi.org/10.2527/asasann.2017.265

Hamel, J., Zhang, Y., Wente, N., & Krömker, V. (2020). Non-S. aureus staphylococci (NAS) in milk samples: Infection or contamination? *Veterinary Microbiology*, *242*, 108594. https://doi.org/10.1016/j.vetmic.2020.108594

Handiso, B., Pääkkönen, T., & Wilson, B. P. (2024). Effect of pyrolysis temperature on the physical and chemical characteristics of pine wood biochar. *Waste Management Bulletin*, *2*(4), 281–287. https://doi.org/10.1016/j.wmb.2024.11.008

Heinonen-Tanski, H., Mohaibes, M., Karinen, P., & Koivunen, J. (2006). Methods to reduce pathogen microorganisms in manure. *Livestock Science*, *102*(3), 248–255. https://doi.org/10.1016/j.livsci.2006.03.024

Heinze, G., & Dunkler, D. (2017). Five myths about variable selection. *Transplant International: Official Journal of the European Society for Organ Transplantation*, *30*(1), 6–10. https://doi.org/10.1111/tri.12895

Hemmati, F., Hosseini, H., Mostashari, P., Aliyeva, A., & Mousavi Khaneghah, A. (2023). Application of molecularly imprinted polymers as the sorbent for extraction of chemical contaminants from milk. *International Journal of Environmental Health Research*, 1–16. https://doi.org/10.1080/09603123.2023.2207484

Hinthong, W., Pumipuntu, N., Santajit, S., Kulpeanprasit, S., Buranasinsup, S., Sookrung, N., Chaicumpa, W., Aiumurai, P., & Indrawattana, N. (2017). Detection and drug resistance profile of *Escherichia coli* from subclinical mastitis cows and water supply in dairy farms in Saraburi Province, Thailand. *PeerJ*, *5*, e3431. https://doi.org/10.7717/peerj.3431

Holmes, A. H., & Sharland, M. (2013). The Chennai Declaration: India's landmark national commitment to antibiotic stewardship demonstrates that "truth alone triumphs." *The Journal of Antimicrobial Chemotherapy*, *68*(7), 1453–1454. https://doi.org/10.1093/jac/dkt062

Holschbach, C. L., Aulik, N., Poulsen, K., & Ollivett, T. L. (2020). Prevalence and temporal trends in antimicrobial resistance of bovine respiratory disease pathogen isolates submitted to the Wisconsin Veterinary Diagnostic Laboratory: 2008–2017. *Journal of Dairy Science*, *103*(10), 9464–9472. https://doi.org/10.3168/jds.2019-17570

Hommels, N. M. C., Ferreira, F. C., van den Borne, B. H. P., & Hogeveen, H. (2021). Antibiotic use and potential economic impact of implementing selective dry cow therapy in large US dairies. *Journal of Dairy Science*, *104*(8), Article 8. https://doi.org/10.3168/jds.2020-20016

Hossain, M. Z., Bahar, M. M., Sarkar, B., Donne, S. W., Ok, Y. S., Palansooriya, K. N., Kirkham, M. B., Chowdhury, S., & Bolan, N. (2020). Biochar and its importance on nutrient dynamics in soil and plant. *Biochar*, *2*(4), 379–420. https://doi.org/10.1007/s42773-020-00065-z

Hu, H.-W., Han, X.-M., Shi, X.-Z., Wang, J.-T., Han, L.-L., Chen, D., & He, J.-Z. (2016). Temporal changes of antibiotic-resistance genes and bacterial communities in two contrasting soils treated with cattle manure. *FEMS Microbiology Ecology*, *92*(2), fiv169. https://doi.org/10.1093/femsec/fiv169

Huang, F., Teng, K., Liu, Y., Wang, T., Xia, T., Yun, F., & Zhong, J. (2022). Nisin Z attenuates lipopolysaccharide-induced mastitis by inhibiting the ERK1/2 and p38 mitogen-activated protein kinase signaling pathways. *Journal of Dairy Science*, *105*(4), 3530–3543. https://doi.org/10.3168/jds.2021-21356

Huang, S., Tian, P., Kou, X., An, N., Wu, Y., Dong, J., Cai, H., Li, B., Xue, Y., Liu, Y., & Ji, H. (2022). The prevalence and characteristics of extended-spectrum β-lactamase *Escherichia coli* in raw milk and dairy farms in Northern Xinjiang, China. *International Journal of Food Microbiology*, *381*, 109908. https://doi.org/10.1016/j.ijfoodmicro.2022.109908

Hutchison, M. L., Walters, L. D., Avery, S. M., Munro, F., & Moore, A. (2005). Analyses of livestock production, waste storage, and pathogen levels and prevalences in farm manures. *Applied and Environmental Microbiology*, *71*(3), 1231–1236. https://doi.org/10.1128/AEM.71.3.1231-1236.2005

Intergovernmental Panel on Climate Change (IPCC). (2023). *Climate Change 2021 – The Physical Science Basis: Working Group I Contribution to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press. https://doi.org/10.1017/9781009157896

Iskandar, K., Murugaiyan, J., Halat, D. H., Hage, S. E., Chibabhai, V., Adukkadukkam, S., Roques, C., Molinier, L., Salameh, P., & Van Dongen, M. (2022). Antibiotic Discovery and Resistance: The Chase and the Race. *Antibiotics*, *11*(2), Article 2. https://doi.org/10.3390/antibiotics11020182

Jackson, C. R., Fedorka-Cray, P. J., & Barrett, J. B. (2004). Use of a Genus- and Species-Specific Multiplex PCR for Identification of Enterococci. *Journal of Clinical Microbiology*, *42*(8), 3558–3565. https://doi.org/10.1128/JCM.42.8.3558-3565.2004

Jacobs, K., Wind, L., Krometis, L.-A., Hession, W. C., & Pruden, A. (2019). Fecal Indicator Bacteria and Antibiotic Resistance Genes in Storm Runoff from Dairy Manure and

Compost-Amended Vegetable Plots. *Journal of Environmental Quality*, *48*(4), 1038–1046. https://doi.org/10.2134/jeq2018.12.0441

Jadeja, N. B., & Worrich, A. (2022). From gut to mud: Dissemination of antimicrobial resistance between animal and agricultural niches. *Environmental Microbiology*, *24*(8), 3290–3306. https://doi.org/10.1111/1462-2920.15927

Jang, H. M., & Kan, E. (2022). Enhanced removal of antibiotic resistance genes and human bacterial pathogens during anaerobic digestion of dairy manure via addition of manure biochar. *Chemosphere*, *304*, 135178. https://doi.org/10.1016/j.chemosphere.2022.135178

Jauregi, L., González, A., Garbisu, C., & Epelde, L. (2023). Organic amendment treatments for antimicrobial resistance and mobile element genes risk reduction in soil-crop systems. *Scientific Reports*, *13*(1), 863. https://doi.org/10.1038/s41598-023-27840-9

Jeppsson, K.-H., Magnusson, M., Nilsson, S. B., Ekman, L., Winblad von Walter, L., Jansson, L.-E., Landin, H., Rosander, A., & Bergsten, C. (2024). Comparisons of recycled manure solids and wood shavings/sawdust as bedding material -implications for animal welfare, herd health, milk quality and bedding costs in Swedish dairy herds. *Journal of Dairy Science*. https://doi.org/10.3168/jds.2023-24192

Ji, Y., Zhao, Z., Jiang, Q., Loor, J. J., Song, L., Ou, H., Liu, M., Sun, C., Feng, X., Lei, L., Han, W., Li, X., & Gu, J. (2023). Potential of phage EF-N13 as an alternative treatment strategy for mastitis infections caused by multidrug-resistant *Enterococcus faecalis*. *Journal of Dairy Science*, *106*(12), 9174–9185. https://doi.org/10.3168/jds.2022-22892

Jia, Y., Mao, W., Liu, B., Zhang, S., Cao, J., & Xu, X. (2022). Study on the drug resistance and pathogenicity of *Escherichia coli* isolated from calf diarrhea and the distribution of virulence genes and antimicrobial resistance genes. *Frontiers in Microbiology*, *13*, 992111. https://doi.org/10.3389/fmicb.2022.992111

Kalińska, A., Jaworski, S., Wierzbicki, M., & Gołębiewski, M. (2019). Silver and Copper Nanoparticles—An Alternative in Future Mastitis Treatment and Prevention? *International Journal of Molecular Sciences*, *20*(7), 1672. https://doi.org/10.3390/ijms20071672

Kalińska, A., Jaworski, S., Wierzbicki, M., Kot, M., Radzikowski, D., Smulski, S., & Gołębiewski, M. (2023). Silver and Copper Nanoparticles as the New Biocidal Agents Used in Pre- and Post-Milking Disinfectants with the Addition of Cosmetic Substrates in Dairy Cows. *International Journal of Molecular Sciences*, *24*(2), 1658. https://doi.org/10.3390/ijms24021658

Kang, Z., Jia, X., Zhang, Y., Kang, X., Ge, M., Liu, D., Wang, C., & He, Z. (2022). A Review on Application of Biochar in the Removal of Pharmaceutical Pollutants through Adsorption and Persulfate-Based AOPs. *Sustainability*, *14*(16), Article 16. https://doi.org/10.3390/su141610128

Kawasaki, M., & Ambrosini, Y. M. (2024). Differential Colonization and Mucus Ultrastructure Visualization in Bovine Ileal and Rectal Organoid-Derived Monolayers Exposed to Enterohemorrhagic Escherichia coli. *International Journal of Molecular Sciences*, *25*(9), Article 9. https://doi.org/10.3390/ijms25094914

Ke, D., Picard, F. J., Martineau, F., Ménard, C., Roy, P. H., Ouellette, M., & Bergeron, M. G. (1999). Development of a PCR assay for rapid detection of enterococci. *Journal of Clinical Microbiology*, *37*(11), 3497–3503. https://doi.org/10.1128/JCM.37.11.3497-3503.1999

Keenum, I., Williams, R. K., Ray, P., Garner, E. D., Knowlton, K. F., & Pruden, A. (2021). Combined effects of composting and antibiotic administration on cattle manure–borne antibiotic resistance genes. *Microbiome*, *9*(1). https://doi.org/10.1186/s40168-021-01006-z

Kernéis, S., Valade, S., & Woerther, P.-L. (2020). Back into the wild: How resistant pathogens become susceptible again? *Intensive Care Medicine*, *46*(2), 361–363. https://doi.org/10.1007/s00134-020-05932-8

Khosa, S., AlKhatib, Z., & Smits, S. H. J. (2013). NSR from Streptococcus agalactiae confers resistance against nisin and is encoded by a conserved nsr operon. *Biological Chemistry*, *394*(11), 1543–1549. https://doi.org/10.1515/hsz-2013-0167

Khurana, S. K., Sehrawat, A., Tiwari, R., Prasad, M., Gulati, B., Shabbir, M. Z., Chhabra, R., Karthik, K., Patel, S. K., Pathak, M., Iqbal Yatoo, Mohd., Gupta, V. K., Dhama, K., Sah, R., & Chaicumpa, W. (2021). Bovine brucellosis – a comprehensive review. *The Veterinary Quarterly*, *41*(1), 61–88. https://doi.org/10.1080/01652176.2020.1868616

Klaas, I. C., & Zadoks, R. N. (2018). An update on environmental mastitis: Challenging perceptions. *Transboundary and Emerging Diseases*, *65*(S1), 166–185. https://doi.org/10.1111/tbed.12704

Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., Goossens, H., & Laxminarayan, R. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(15), E3463–E3470. https://doi.org/10.1073/pnas.1717295115

Klima, C. L., Zaheer, R., Cook, S. R., Booker, C. W., Hendrick, S., Alexander, T. W., & McAllister, T. A. (2014). Pathogens of Bovine Respiratory Disease in North American Feedlots Conferring Multidrug Resistance via Integrative Conjugative Elements. *Journal of Clinical Microbiology*, *52*(2), 438–448. https://doi.org/10.1128/JCM.02485-13

Klobucar, K., & Brown, E. D. (2022). New potentiators of ineffective antibiotics: Targeting the Gram-negative outer membrane to overcome intrinsic resistance. *Current Opinion in Chemical Biology*, *66*, 102099. https://doi.org/10.1016/j.cbpa.2021.102099

Kolenda, R., Burdukiewicz, M., & Schierack, P. (2015). A systematic review and meta-analysis of the epidemiology of pathogenic *Escherichia coli* of calves and the role of calves as reservoirs for human pathogenic *E. coli*. *Frontiers in Cellular and Infection Microbiology*, *5*, 23. https://doi.org/10.3389/fcimb.2015.00023

Kot, M., Kalińska, A., Jaworski, S., Wierzbicki, M., Smulski, S., & Gołębiewski, M. (2023). In Vitro Studies of Nanoparticles as a Potentially New Antimicrobial Agent for the Prevention and Treatment of Lameness and Digital Dermatitis in Cattle. *International Journal of Molecular Sciences*, *24*(7), 6146. https://doi.org/10.3390/ijms24076146

Laconi, A., Mughini-Gras, L., Tolosi, R., Grilli, G., Trocino, A., Carraro, L., Di Cesare, F., Cagnardi, P., & Piccirillo, A. (2021). Microbial community composition and antimicrobial resistance in agricultural soils fertilized with livestock manure from conventional farming in Northern Italy. *Science of the Total Environment*, *760*. https://doi.org/10.1016/j.scitotenv.2020.143404

Leach, K. A., Archer, S. C., Breen, J. E., Green, M. J., Ohnstad, I. C., Tuer, S., & Bradley, A. J. (2015). Recycling manure as cow bedding: Potential benefits and risks for UK dairy farms. *The Veterinary Journal*, *206*(2), 123–130. https://doi.org/10.1016/j.tvjl.2015.08.013

Lee, J. H. (2019). Perspectives towards antibiotic resistance: From molecules to population. *Journal of Microbiology*, *57*(3), 181–184. https://doi.org/10.1007/s12275-019-0718-8

Li, H., Lin, L., Peng, Y., Hao, Y., Li, Z., Li, J., Yu, M., Li, X., Lu, Y., Gu, W., & Zhang, B. (2024). Biochar's dual role in greenhouse gas emissions: Nitrogen fertilization dependency and mitigation potential. *The Science of the Total Environment*, *917*, 170293. https://doi.org/10.1016/j.scitotenv.2024.170293

Li, J., Lu, H., Yang, H., Wen, X., Huang, Y., & Li, Q. (2023). Performances of antibiotic resistance genes profile upon the action of biochar-activated peroxydisulfate in composting. *Journal of Environmental Management*, *334*, 117509. https://doi.org/10.1016/j.jenvman.2023.117509

Li, L., Guo, C., Ai, L., Dou, C., Wang, G., & Sun, H. (2014). Research on degradation of penicillins in milk by β-lactamase using ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry. *Journal of Dairy Science*, *97*(7), 4052–4061. https://doi.org/10.3168/jds.2014-7952

Li, S., Yao, Q., Liu, J., Wei, D., Zhou, B., Zhu, P., Cui, X., Jin, J., Liu, X., & Wang, G. (2020). Profiles of antibiotic resistome with animal manure application in black soils of northeast China. *Journal of Hazardous Materials*, *384*. https://doi.org/10.1016/j.jhazmat.2019.121216

Lim, M. S. M., & Grohn, Y. T. (2021). Comparison of China's and the European Union's Approaches to Antimicrobial Stewardship in the Pork Industry. *Foodborne Pathogens and Disease*. https://doi.org/10.1089/fpd.2020.2887

Liu, G., Ali, T., Gao, J., ur Rahman, S., Yu, D., Barkema, H. W., Huo, W., Xu, S., Shi, Y., Kastelic, J. P., & Han, B. (2020). Co-Occurrence of Plasmid-Mediated Colistin Resistance (*mcr-1*) and Extended-Spectrum *β* -Lactamase Encoding Genes in *Escherichia coli* from Bovine Mastitic Milk in China. *Microbial Drug Resistance*, *26*(6), 685–696. https://doi.org/10.1089/mdr.2019.0333

Liu, M., Liu, C., Liao, W., Xie, J., Zhang, X., & Gao, Z. (2021). Impact of biochar application on gas emissions from liquid pig manure storage. *The Science of the Total Environment*, *771*, 145454. https://doi.org/10.1016/j.scitotenv.2021.145454

Lobanovska, M., & Pilla, G. (2017). Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? *The Yale Journal of Biology and Medicine*, *90*(1), 135–145.

Lyu, H., Gong, Y., Tang, J., Huang, Y., & Wang, Q. (2016). Immobilization of heavy metals in electroplating sludge by biochar and iron sulfide. *Environmental Science and Pollution Research*, *23*(14), 14472–14488. https://doi.org/10.1007/s11356-016-6621-5

Ma, S., Shen, Y., Ding, J., Cheng, H., Zhou, H., Ge, M., Wang, J., Cheng, Q., Zhang, D., Zhang, Y., Xu, P., & Zhang, P. (2024). Effects of biochar and volcanic rock addition on humification and microbial community during aerobic composting of cow manure. *Bioresource Technology*, *391*, 129973. https://doi.org/10.1016/j.biortech.2023.129973

Ma, Y., Khan, M. Z., Xiao, J., Alugongo, G. M., Chen, X., Li, S., Wang, Y., & Cao, Z. (2022). An Overview of Waste Milk Feeding Effect on Growth Performance, Metabolism, Antioxidant Status and Immunity of Dairy Calves. *Frontiers in Veterinary Science*, *9*. https://www.frontiersin.org/articles/10.3389/fvets.2022.898295

Mader, R., Demay, C., Jouvin-Marche, E., Ploy, M.-C., Barraud, O., Bernard, S., Lacotte, Y., Pulcini, C., Weinbach, J., Berling, C., Bouqueau, M., Hlava, A., Habl, C., Kernstock, E., Strauss, R., Muchl, R., Buhmann, V., Versporten, A., Ingenbleek, A., … Madec, J.-Y. (2022). Defining the scope of the European Antimicrobial Resistance Surveillance network in Veterinary medicine (EARS-Vet): A bottom-up and One Health approach. *Journal of Antimicrobial Chemotherapy*, *77*(3), 816–826. https://doi.org/10.1093/jac/dkab462

Magiorakos, A.-P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T., & Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, *18*(3), 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x

Marutescu, L. G., Jaga, M., Postolache, C., Barbuceanu, F., Milita, N. M., Romascu, L. M., Schmitt, H., De Roda Husman, A. M., Sefeedpari, P., Glaeser, S., Kämpfer, P., Boerlin, P., Topp, E., Gradisteanu Pircalabioru, G., Chifiriuc, M. C., & Popa, M. (2022). Insights into the impact of manure on the environmental antibiotic residues and resistance pool. *Frontiers in Microbiology*, *13*, 965132. https://doi.org/10.3389/fmicb.2022.965132

Maynou, G., Bach, A., & Terré, M. (2017). Feeding of waste milk to Holstein calves affects antimicrobial resistance of *Escherichia coli* and *Pasteurella multocida* isolated from fecal and nasal swabs. *Journal of Dairy Science*, *100*(4), 2682–2694. https://doi.org/10.3168/jds.2016-11891

Maynou, G., Migura-Garcia, L., Chester-Jones, H., Ziegler, D., Bach, A., & Terré, M. (2017). Effects of feeding pasteurized waste milk to dairy calves on phenotypes and genotypes of antimicrobial resistance in fecal *Escherichia coli* isolates before and after weaning. *Journal of Dairy Science*, *100*(10), 7967–7979. https://doi.org/10.3168/jds.2017-13040

Mazurkiewicz-Pisarek, A., Baran, J., & Ciach, T. (2023). Antimicrobial Peptides: Challenging Journey to the Pharmaceutical, Biomedical, and Cosmeceutical Use. *International Journal of Molecular Sciences*, *24*(10), 9031. https://doi.org/10.3390/ijms24109031

McCubbin, K. D., de Jong, E., Lam, T. J. G. M., Kelton, D. F., Middleton, J. R., McDougall, S., De Vliegher, S., Godden, S., Rajala-Schultz, P. J., Rowe, S., Speksnijder, D. C., Kastelic, J. P., & Barkema, H. W. (2022). Invited review: Selective use of antimicrobials in dairy cattle at drying-off. *Journal of Dairy Science*, *105*(9), 7161–7189. https://doi.org/10.3168/jds.2021-21455

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Mena-Bueno, S., Poveda-Urkixo, I., Asensio, D., Echarte, I., Zabalza-Baranguá, A., & Grilló, M. J. (2022). BruSIC: A novel selective medium for the primary isolation of *Brucella* in veterinary samples. *Microbiology Spectrum*, *10*(6), e01759-22. https://doi.org/10.1128/spectrum.01759-22

Menz, J., Olsson, O., & Kümmerer, K. (2019). Antibiotic residues in livestock manure: Does the EU risk assessment sufficiently protect against microbial toxicity and selection of resistant bacteria in the environment? *Journal of Hazardous Materials*, *379*, 120807. https://doi.org/10.1016/j.jhazmat.2019.120807

Messele, Y. E., Hasoon, M. F., Trott, D. J., Veltman, T., McMeniman, J. P., Kidd, S. P., Low, W. Y., & Petrovski, K. R. (2022). Longitudinal Analysis of Antimicrobial Resistance

among Enterococcus Species Isolated from Australian Beef Cattle Faeces at Feedlot Entry and Exit. *Animals*, *12*(19), Article 19. https://doi.org/10.3390/ani12192690

Meyer, S., Glaser, B., & Quicker, P. (2011, October 21). Technical, Economical, and Climate-Related Aspects of Biochar Production Technologies: A Literature Review. *ACS Publications*; American Chemical Society. https://doi.org/10.1021/es201792c

Mohammadian, F., Rahmani, H. K., Bidarian, B., & Khoramian, B. (2022). Isolation and evaluation of the efficacy of bacteriophages against multidrug-resistant (MDR), methicillin-resistant (MRSA) and biofilm-producing strains of *Staphylococcus aureus* recovered from bovine mastitis. *BMC Veterinary Research*, *18*(1), 406. https://doi.org/10.1186/s12917-022-03501-3

Mohammadi-Aragh, M. K., Linhoss, J. E., Marty, C. A., Evans, J. D., Purswell, J. L., Chaves-Cordoba, B., Chesser, G. D., & Lowe, J. W. (2022). Evaluating the Effects of Pine and Miscanthus Biochar on *Escherichia coli*, Total Aerobic Bacteria, and Bacterial Communities in Commercial Broiler Litter. *Avian Diseases*, *66*(4), 1–10. https://doi.org/10.1637/aviandiseases-D-22-00056

Mshana, S. E., Sindato, C., Matee, M. I., & Mboera, L. E. G. (2021). Antimicrobial use and resistance in agriculture and food production systems in africa: A systematic review. *Antibiotics*, *10*(8). https://doi.org/10.3390/antibiotics10080976

Munita, J. M., & Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiology Spectrum*, *4*(2), 4.2.15. https://doi.org/10.1128/microbiolspec.VMBF-0016-2015

NAHMS. (2018). *Dairy 2014–Health and management practices on U.S. dairy operations, 2014. USDA, Animal and Plant Health Inspection Service, Veterinary Services, Center for Epidemiology and Animal Health, National Animal Health Monitoring System. Accessed*.
https://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy14/Dairy14_dr_PartIII.pdf

Nakada, S., Fujimoto, Y., Kohara, J., & Makita, K. (2023). Economic losses associated with mastitis due to bovine leukemia virus infection. *Journal of Dairy Science*, *106*(1), 576–588. https://doi.org/10.3168/jds.2021-21722

Naranjo-Lucena, A., & Slowey, R. (2023). Invited review: Antimicrobial resistance in bovine mastitis pathogens: A review of genetic determinants and prevalence of resistance in European countries. *Journal of Dairy Science*, *106*(1), 1–23. https://doi.org/10.3168/jds.2022-22267

Obaidat, M. M., Bani Salman, A. E., Davis, M. A., & Roess, A. A. (2018). Major diseases, extensive misuse, and high antimicrobial resistance of *Escherichia coli* in large- and small-scale dairy cattle farms in Jordan. *Journal of Dairy Science*, *101*(3), 2324–2334. https://doi.org/10.3168/jds.2017-13665

Olasoju, M. I., Olasoju, T. I., Adebowale, O. O., & Adetunji, V. O. (2021). Knowledge and practice of cattle handlers on antibiotic residues in meat and milk in Kwara State, Northcentral Nigeria. *PLOS ONE*, *16*(10), e0257249. https://doi.org/10.1371/journal.pone.0257249

Oliveira, M., Tavares, M., Gomes, D., Touret, T., São Braz, B., Tavares, L., & Semedo-Lemsaddek, T. (2016). Virulence traits and antibiotic resistance among enterococci isolated from dogs with periodontal disease. *Comparative Immunology, Microbiology and Infectious Diseases*, *46*, 27–31. https://doi.org/10.1016/j.cimid.2016.04.002

Oliver, J. P., Gooch, C. A., Lansing, S., Schueler, J., Hurst, J. J., Sassoubre, L., Crossette, E. M., & Aga, D. S. (2020). Invited review: Fate of antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes in US dairy manure management systems. *Journal of Dairy Science*, *103*(2), 1051–1071. https://doi.org/10.3168/jds.2019-16778

Oliver, S. P., Murinda, S. E., & Jayarao, B. M. (2011). Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: A comprehensive review. *Foodborne Pathogens and Disease*, *8*(3), 337–355. https://doi.org/10.1089/fpd.2010.0730

Patwa, D., Bordoloi, U., Dubey, A. A., Ravi, K., Sekharan, S., & Kalita, P. (2022). Energy-efficient biochar production for thermal backfill applications. *The Science of the Total Environment*, *833*, 155253. https://doi.org/10.1016/j.scitotenv.2022.155253

Penati, M., Sala, G., Biscarini, F., Boccardo, A., Bronzo, V., Castiglioni, B., Cremonesi, P., Moroni, P., Pravettoni, D., & Addis, M. F. (2021). Feeding Pre-weaned Calves With Waste Milk Containing Antibiotic Residues Is Related to a Higher Incidence of Diarrhea and Alterations in the Fecal Microbiota. *Frontiers in Veterinary Science*, *8*, 650150. https://doi.org/10.3389/fvets.2021.650150

Peng, S., Feng, Y., Wang, Y., Guo, X., Chu, H., & Lin, X. (2017). Prevalence of antibiotic resistance genes in soils after continually applied with different manure for 30 years. *Journal of Hazardous Materials*, *340*, 16–25. https://doi.org/10.1016/j.jhazmat.2017.06.059

Pereira, A. R., Paranhos, A. G. de O., de Aquino, S. F., & Silva, S. de Q. (2021). Distribution of genetic elements associated with antibiotic resistance in treated and untreated animal husbandry waste and wastewater. *Environmental Science and Pollution Research*, *28*(21), 26380–26403. https://doi.org/10.1007/s11356-021-13784-y

Perez-Mercado, L. F., Lalander, C., Joel, A., Ottoson, J., Dalahmeh, S., & Vinnerås, B. (2019). Biochar filters as an on-farm treatment to reduce pathogens when irrigating with wastewater-polluted sources. *Journal of Environmental Management*, *248*, 109295. https://doi.org/10.1016/j.jenvman.2019.109295

Pires, A. J., Esteves, A. F., Pereira, G., Geraldes, C., Guerreiro, J. F., Abreu, R., Magalhães, R., Chambel, L., Silva, E., Fangueiro, D., Oliveira, M., & Bexiga, R. (2025).

Potential of Pine Biochar to Mitigate Bacterial Hazards Present in Recycled Manure Solids from Dairy Cows. *Veterinary Sciences*, *12*(1), Article 1. https://doi.org/10.3390/vetsci12010043

Pires, A. J., Pereira, G., Fangueiro, D., Bexiga, R., & Oliveira, M. (2024). When the solution becomes the problem: A review on antimicrobial resistance in dairy cattle. *Future Microbiology*. https://www.tandfonline.com/doi/abs/10.2217/fmb-2023-0232

Prado, J., Chieppe, J., Raymundo, A., & Fangueiro, D. (2020). Bio-acidification and enhanced crusting as an alternative to sulphuric acid addition to slurry to mitigate ammonia and greenhouse gases emissions during short term storage. *Journal of Cleaner Production*, *263*, 121443. https://doi.org/10.1016/j.jclepro.2020.121443

Pu, C., Yu, Y., Diao, J., Gong, X., Li, J., & Sun, Y. (2019). Exploring the persistence and spreading of antibiotic resistance from manure to biocompost, soils and vegetables. *Science of the Total Environment*, *688*, 262–269. https://doi.org/10.1016/j.scitotenv.2019.06.081

Qian, X., Gu, J., Sun, W., Wang, X. J., Su, J. Q., & Stedfeld, R. (2018). Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting. *Journal of Hazardous Materials*, *344*, 716–722. https://doi.org/10.1016/j.jhazmat.2017.11.020

Qian, X., Sun, W., Gu, J., Wang, X.-J., Zhang, Y.-J., Duan, M.-L., Li, H.-C., & Zhang, R.-R. (2016). Reducing antibiotic resistance genes, integrons, and pathogens in dairy manure by continuous thermophilic composting. *Bioresource Technology*, *220*, 425–432. https://doi.org/10.1016/j.biortech.2016.08.101

Rajala-Schultz, P., Nødtvedt, A., Halasa, T., & Persson Waller, K. (2021). Prudent Use of Antibiotics in Dairy Cows: The Nordic Approach to Udder Health. *Frontiers in Veterinary Science*, *8*, 623998. https://doi.org/10.3389/fvets.2021.623998

Ramos, S., Silva, V., Dapkevicius, M. de L. E., Igrejas, G., & Poeta, P. (2020). Enterococci, from Harmless Bacteria to a Pathogen. *Microorganisms*, *8*(8), Article 8. https://doi.org/10.3390/microorganisms8081118

Rapp, D., Ross, C., Cave, V., Maclean, P., Jauregui, R., & Brightwell, G. (2023). Medium-term storage of calf beddings affects bacterial community and effectiveness to inactivate zoonotic bacteria. *PloS One*, *18*(12), e0295843. https://doi.org/10.1371/journal.pone.0295843

Regueiro, I., Coutinho, J., Gioelli, F., Balsari, P., Dinuccio, E., & Fangueiro, D. (2016). Acidification of raw and co-digested pig slurries with alum before mechanical separation reduces gaseous emission during storage of solid and liquid fractions. *Agriculture, Ecosystems & Environment*, *227*, 42–51. https://doi.org/10.1016/j.agee.2016.04.016

Rezanejad, M., Karimi, S., & Momtaz, H. (2019). Phenotypic and molecular characterization of antimicrobial resistance in Trueperella pyogenes strains isolated from bovine mastitis and metritis. *BMC Microbiology*, *19*(1), 305. https://doi.org/10.1186/s12866-019-1630-4

Richardson, E. J., Bacigalupe, R., Harrison, E. M., Weinert, L. A., Lycett, S., Vrieling, M., Robb, K., Hoskisson, P. A., Holden, M. T. G., Feil, E. J., Paterson, G. K., Tong, S. Y. C., Shittu, A., Van Wamel, W., Aanensen, D. M., Parkhill, J., Peacock, S. J., Corander, J., Holmes, M., & Fitzgerald, J. R. (2018). Gene exchange drives the ecological success of a multi-host bacterial pathogen. *Nature Ecology & Evolution*, *2*(9), 1468–1478. https://doi.org/10.1038/s41559-018-0617-0

Riley, J. C. (2005). Estimates of Regional and Global Life Expectancy, 1800–2001. *Population and Development Review*, *31*(3), 537–543. https://doi.org/10.1111/j.1728-4457.2005.00083.x

Rodrigues, J., Alvarenga, P., Silva, A. C., Brito, L., Tavares, J., & Fangueiro, D. (2021). Animal Slurry Sanitization through pH Adjustment: Process Optimization and Impact on Slurry Characteristics. *Agronomy*, *11*(3), Article 3. https://doi.org/10.3390/agronomy11030517

Rowbotham, R. F., & Ruegg, P. L. (2016). Bacterial counts on teat skin and in new sand, recycled sand, and recycled manure solids used as bedding in freestalls. *Journal of Dairy Science*, *99*(8), 6594–6608. https://doi.org/10.3168/jds.2015-10674

Rowe, S., Kabera, F., Dufour, S., Godden, S., Roy, J.-P., & Nydam, D. (2023). Selective dry-cow therapy can be implemented successfully in cows of all milk production levels. *Journal of Dairy Science*, *106*(3), 1953–1967. https://doi.org/10.3168/jds.2022-22547

Rowe, S. M., Godden, S. M., Nydam, D. V., Gorden, P. J., Lago, A., Vasquez, A. K., Royster, E., Timmerman, J., & Thomas, M. J. (2020). Randomized controlled non-inferiority trial investigating the effect of 2 selective dry-cow therapy protocols on antibiotic use at dry-off and dry period intramammary infection dynamics. *Journal of Dairy Science*, *103*(7), 6473–6492. https://doi.org/10.3168/jds.2019-17728

Rowe, S. M., Vasquez, A. K., Godden, S. M., Nydam, D. V., Royster, E., Timmerman, J., & Boyle, M. (2021). Evaluation of 4 predictive algorithms for intramammary infection status in late-lactation cows. *Journal of Dairy Science*, *104*(10), 11035–11046. https://doi.org/10.3168/jds.2021-20504

Ruuskanen, M., Muurinen, J., Meierjohan, A., Pärnänen, K., Tamminen, M., Lyra, C., Kronberg, L., & Virta, M. (2016). Fertilizing with Animal Manure Disseminates Antibiotic Resistance Genes to the Farm Environment. *Journal of Environmental Quality*, *45*(2), 488–493. https://doi.org/10.2134/jeq2015.05.0250

Saitoh, T., & Shibayama, T. (2016). Removal and degradation of β-lactam antibiotics in water using didodecyldimethylammonium bromide-modified montmorillonite organoclay. *Journal of Hazardous Materials*, *317*, 677–685. https://doi.org/10.1016/j.jhazmat.2016.06.003

Sapkota, S., Ghimire, R., Bista, P., Hartmann, D., Rahman, T., & Adhikari, S. (2024). Greenhouse gas mitigation and soil carbon stabilization potential of forest biochar varied with biochar type and characteristics. *Science of The Total Environment*, *931*, 172942. https://doi.org/10.1016/j.scitotenv.2024.172942

Schmieder, R., & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics*, *27*(6), 863–864. https://doi.org/10.1093/bioinformatics/btr026

Schouler, C., Schaeffer, B., Brée, A., Mora, A., Dahbi, G., Biet, F., Oswald, E., Mainil, J., Blanco, J., & Moulin-Schouleur, M. (2012). Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. *Journal of Clinical Microbiology*, *50*(5), 1673–1678. https://doi.org/10.1128/JCM.05057-11

Sears, P. M., Smith, B. S., Stewart, W. K., Gonzalez, R. N., Rubino, S. D., Gusik, S. A., Kulisek, E. S., Projan, S. J., & Blackburn, P. (1992). Evaluation of a Nisin-Based Germicidal Formulation on Teat Skin of Live Cows. *Journal of Dairy Science*, *75*(11), 3185–3190. https://doi.org/10.3168/jds.S0022-0302(92)78083-7

Semedo-Lemsaddek, T., Nóbrega, C. S., Ribeiro, T., Pedroso, N. M., Sales-Luís, T., Lemsaddek, A., Tenreiro, R., Tavares, L., Vilela, C. L., & Oliveira, M. (2013). Virulence traits and antibiotic resistance among enterococci isolated from Eurasian otter (*Lutra lutra*). *Veterinary Microbiology*, *163*(3–4), 378–382. https://doi.org/10.1016/j.vetmic.2012.12.032

Sharma, C., Rokana, N., Chandra, M., Singh, B. P., Gulhane, R. D., Gill, J. P. S., Ray, P., Puniya, A. K., & Panwar, H. (2017). Antimicrobial Resistance: Its Surveillance, Impact, and Alternative Management Strategies in Dairy Animals. *Frontiers in Veterinary Science*, *4*, 237. https://doi.org/10.3389/fvets.2017.00237

Sharma, D., Manimaran, A., Kumaresan, A., Sivaram, M., & Rajendran, D. (2022). Antimicrobials use and their indications in dairy farm and individual farmer production conditions in southern India. *Tropical Animal Health and Production*, *54*(1), 29. https://doi.org/10.1007/s11250-021-03025-2

Shrestha, R. K., Jacinthe, P., Lal, R., Lorenz, K., Singh, M. P., Demyan, S. M., Ren, W., & Lindsey, L. E. (2023). Biochar as a negative emission technology: A synthesis of field research on greenhouse gas emissions. *Journal of Environmental Quality*, *52*(4), 769–798. https://doi.org/10.1002/jeq2.20475

Silva, T. E. da, & Cabrera, V. E. (2024). The DairyPrint Model: A Decision-Support Model to Help Dairy Farmers and Other Stakeholders Towards Improved Sustainability. *Journal of Dairy Science*, *0*(0). https://doi.org/10.3168/jds.2024-24946

Silva, E., Leitão, S., Tenreiro, T., Pomba, C., Nunes, T., Lopes Da Costa, L., & Mateus, L. (2009). Genomic and phenotypic characterization of *Escherichia coli* isolates recovered from the uterus of puerperal dairy cows. *Journal of Dairy Science*, *92*(12), 6000–6010. https://doi.org/10.3168/jds.2009-2358

Singh, S. K., Ekka, R., Mishra, M., & Mohapatra, H. (2017). Association study of multiple antibiotic resistance and virulence: A strategy to assess the extent of risk posed by bacterial population in aquatic environment. *Environmental Monitoring and Assessment*, *189*(7), 320. https://doi.org/10.1007/s10661-017-6005-4

Solomon, E. B., Yaron, S., & Matthews, K. R. (2002). Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Applied and Environmental Microbiology*, *68*(1), 397–400. https://doi.org/10.1128/AEM.68.1.397-400.2002

Son, E.-B., Poo, K.-M., Chang, J.-S., & Chae, K.-J. (2018). Heavy metal removal from aqueous solutions using engineered magnetic biochars derived from waste marine macro-algal biomass. *Science of The Total Environment*, *615*, 161–168. https://doi.org/10.1016/j.scitotenv.2017.09.171

Sørensen, P., & Eriksen, J. (2009). Effects of slurry acidification with sulphuric acid combined with aeration on the turnover and plant availability of nitrogen. *Agriculture, Ecosystems & Environment*, *131*(3), 240–246. https://doi.org/10.1016/j.agee.2009.01.024

Sorter, D. E., Kester, H. J., & Hogan, J. S. (2014). Short communication: Bacterial counts in recycled manure solids bedding replaced daily or deep packed in freestalls. *Journal of Dairy Science*, *97*(5), 2965–2968. https://doi.org/10.3168/jds.2013-7814

Stevens, M., Piepers, S., & De Vliegher, S. (2019). The effect of mastitis management input and implementation of mastitis management on udder health, milk quality, and antimicrobial consumption in dairy herds. *Journal of Dairy Science*, *102*(3), 2401–2415. https://doi.org/10.3168/jds.2018-15237

Stortchevoi, A., Kamelamela, N., & Levine, S. S. (2020). SPRI Beads-based Size Selection in the Range of 2-10kb. *Journal of Biomolecular Techniques : JBT*, *31*(1), 7–10. https://doi.org/10.7171/jbt.20-3101-002

Sukhum, K. V., Vargas, R. C., Boolchandani, M., D'Souza, A. W., Patel, S., Kesaraju, A., Walljasper, G., Hegde, H., Ye, Z., Valenzuela, R. K., Gunderson, P., Bendixsen, C., Dantas, G., & Shukla, S. K. (2021). Manure Microbial Communities and Resistance Profiles Reconfigure after Transition to Manure Pits and Differ from Those in Fertilized Field Soil. *mBio*, *12*(3), e00798-21. https://doi.org/10.1128/mBio.00798-21

Tabaran, A., Soulageon, V., Chirila, F., Reget, O. L., Mihaiu, M., Borzan, M., & Dan, S. D. (2022). Pathogenic E. coli from Cattle as a Reservoir of Resistance Genes to Various Groups of Antibiotics. *Antibiotics*, *11*(3), 404. https://doi.org/10.3390/antibiotics11030404

Tang, K. L., Caffrey, N. P., Nóbrega, D. B., Cork, S. C., Ronksley, P. E., Barkema, H. W., Polachek, A. J., Ganshorn, H., Sharma, N., Kellner, J. D., & Ghali, W. A. (2017). Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: A systematic review and meta-analysis. *The Lancet. Planetary Health*, *1*(8), e316–e327. https://doi.org/10.1016/S2542-5196(17)30141-9

Tark, D.-S., Moon, D. C., Kang, H. Y., Kim, S.-R., Nam, H.-M., Lee, H.-S., Jung, S.-C., & Lim, S.-K. (2017). Antimicrobial susceptibility and characterization of extended-spectrum β-lactamases in *Escherichia coli* isolated from bovine mastitic milk in South Korea from 2012 to 2015. *Journal of Dairy Science*, *100*(5), 3463–3469. https://doi.org/10.3168/jds.2016-12276

Teklemariam, A. D., Al Hindi, R., Qadri, I., Alharbi, M. G., Hashem, A. M., Alrefaei, A. A., Basamad, N. A., Haque, S., Alamri, T., & Harakeh, S. (2023). Phage cocktails – an emerging approach for the control of bacterial infection with major emphasis on foodborne pathogens. *Biotechnology and Genetic Engineering Reviews*, 1–29. https://doi.org/10.1080/02648725.2023.2178870

The European Green Deal (2019). https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52019DC0640

Thomsen, P. T., Shearer, J. K., & Houe, H. (2023). Prevalence of lameness in dairy cows: A literature review. *The Veterinary Journal*, *295*, 105975. https://doi.org/10.1016/j.tvjl.2023.105975

Tiseo, K., Huber, L., Gilbert, M., Robinson, T. P., & Van Boeckel, T. P. (2020). Global Trends in Antimicrobial Use in Food Animals from 2017 to 2030. *Antibiotics*, *9*(12), 918. https://doi.org/10.3390/antibiotics9120918

Titouche, Y., Akkou, M., Houali, K., Auvray, F., & Hennekinne, J. (2022). Role of milk and milk products in the spread of methicillin-resistant *Staphylococcus aureus* in the dairy production chain. *Journal of Food Science*, *87*(9), 3699–3723. https://doi.org/10.1111/1750-3841.16259

Titze, I., Lehnherr, T., Lehnherr, H., & Krömker, V. (2020). Efficacy of Bacteriophages Against *Staphylococcus aureus* Isolates from Bovine Mastitis. *Pharmaceuticals*, *13*(3), 35. https://doi.org/10.3390/ph13030035

Tommasoni, C., Fiore, E., Lisuzzo, A., & Gianesella, M. (2023). Mastitis in Dairy Cattle: On-Farm Diagnostics and Future Perspectives. *Animals*, *13*(15), Article 15. https://doi.org/10.3390/ani13152538

Tripathi, N., & Goshisht, M. K. (2022). Recent Advances and Mechanistic Insights into Antibacterial Activity, Antibiofilm Activity, and Cytotoxicity of Silver Nanoparticles. *ACS Applied Bio Materials*, *5*(4), 1391–1463. https://doi.org/10.1021/acsabm.2c00014

US-EPA, E. (2006). Global anthropogenic non-CO2 greenhouse gas emissions: 1990–2020. *Appendix AD. Washington, DC, USA: United States Environmental Protection Agency. Electricity (Heat) Supply*.

Van Boeckel, T. P., Glennon, E. E., Chen, D., Gilbert, M., Robinson, T. P., Grenfell, B. T., Levin, S. A., Bonhoeffer, S., & Laxminarayan, R. (2017). Reducing antimicrobial use in food animals. *Science (New York, N.y.)*, *351*(6358), 1350–1352. https://doi.org/10.1126/science.aao1495

Van Boeckel, T. P., Pires, J., Silvester, R., Zhao, C., Song, J., Criscuolo, N. G., Gilbert, M., Bonhoeffer, S., & Laxminarayan, R. (2019). Global trends in antimicrobial resistance in animals in low- and middle-income countries. *Science*, *365*(6459), eaaw1944. https://doi.org/10.1126/science.aaw1944

Van Metre, D. C. (2017). Pathogenesis and Treatment of Bovine Foot Rot. *Veterinary Clinics of North America: Food Animal Practice*, *33*(2), 183–194. https://doi.org/10.1016/j.cvfa.2017.02.003

Varma, V. S., Parajuli, R., Scott, E., Canter, T., Lim, T. T., Popp, J., & Thoma, G. (2021). Dairy and swine manure management—Challenges and perspectives for sustainable treatment technology. *The Science of the Total Environment*, *778*, 146319. https://doi.org/10.1016/j.scitotenv.2021.146319

Vasiliev, G., Kubo, A.-L., Vija, H., Kahru, A., Bondar, D., Karpichev, Y., & Bondarenko, O. (2023). Synergistic antibacterial effect of copper and silver nanoparticles and their mechanism of action. *Scientific Reports*, *13*(1), 9202. https://doi.org/10.1038/s41598-023-36460-2

Vieira Firmino, M., Trémier, A., Couvert, A., & Szymczyk, A. (2024). New insights into biochar ammoniacal nitrogen adsorption and its correlation to aerobic degradation ammonia emissions. *Waste Management*, *178*, 257–266. https://doi.org/10.1016/j.wasman.2024.02.032

Vos, T., Lim, S. S., Abbafati, C., Abbas, K. M., Abbasi, M., Abbasifard, M., Abbasi-Kangevari, M., Abbastabar, H., Abd-Allah, F., Abdelalim, A., Abdollahi, M., Abdollahpour, I., Abolhassani, H., Aboyans, V., Abrams, E. M., Abreu, L. G., Abrigo, M. R. M., Abu-Raddad, L. J., Abushouk, A. I., … Murray, C. J. L. (2020). Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: A systematic analysis for the Global Burden of Disease Study 2019. *The Lancet*, *396*(10258), 1204–1222. https://doi.org/10.1016/S0140-6736(20)30925-9

Wang, G., Li, X., & Wang, Z. (2016). APD3: The antimicrobial peptide database as a tool for research and education. *Nucleic Acids Research*, *44*(D1), D1087-1093. https://doi.org/10.1093/nar/gkv1278

Wang, M.-C., Fan, Y.-H., Zhang, Y.-Z., Bregente, C. J. B., Lin, W.-H., Chen, C.-A., Lin, T.-P., & Kao, C.-Y. (2023). Characterization of uropathogenic *Escherichia coli* phylogroups associated with antimicrobial resistance, virulence factor distribution, and virulence-related phenotypes. *Infection, Genetics and Evolution*, *114*, 105493. https://doi.org/10.1016/j.meegid.2023.105493

Welsh, R. D., Dye, L. B., Payton, M. E., & Confer, A. W. (2004). Isolation and Antimicrobial Susceptibilities of Bacterial Pathogens from Bovine Pneumonia: 1994–2002. *Journal of Veterinary Diagnostic Investigation*, *16*(5), 426–431. https://doi.org/10.1177/104063870401600510

Willems, R. P. J., van Dijk, K., Vehreschild, M. J. G. T., Biehl, L. M., Ket, J. C. F., Remmelzwaal, S., & Vandenbroucke-Grauls, C. M. J. E. (2023). Incidence of infection with multidrug-resistant Gram-negative bacteria and vancomycin-resistant enterococci in carriers: A systematic review and meta-regression analysis. *The Lancet Infectious Diseases*, *23*(6), 719–731. https://doi.org/10.1016/S1473-3099(22)00811-8

Wilson, D. J., Pempek, J. A., Cheng, T.-Y., Habing, G., Proudfoot, K. L., Winder, C. B., & Renaud, D. L. (2023). A survey of male and female dairy calf care practices and opportunities for change. *Journal of Dairy Science*, *106*(1), Article 1. https://doi.org/10.3168/jds.2022-22238

Winder, C. B., Sargeant, J. M., Kelton, D. F., Leblanc, S. J., Duffield, T. F., Glanville, J., Wood, H., Churchill, K. J., Dunn, J., Bergevin, M. D., Dawkins, K., Meadows, S., & O'Connor, A. M. (2019). Comparative efficacy of blanket versus selective dry-cow therapy: A systematic review and pairwise meta-analysis. *Animal Health Research Reviews*, *20*(2), 217–228. https://doi.org/10.1017/S1466252319000306

Wood, D. E., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology*, *20*(1), 257. https://doi.org/10.1186/s13059-019-1891-0

Woolhouse, M., Ward, M., van Bunnik, B., & Farrar, J. (2015). Antimicrobial resistance in humans, livestock and the wider environment. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*(1670), 20140083. https://doi.org/10.1098/rstb.2014.0083

World Health Organization. (2015). *Global action plan on antimicrobial resistance*. World Health Organization. https://iris.who.int/handle/10665/193736

Wu, G., Tham, P. E., Chew, K. W., Munawaroh, H. S. H., Tan, I. S., Wan-Mohtar, W. A. A. Q. I., Sriariyanun, M., & Show, P. L. (2023). Net zero emission in circular bioeconomy

from microalgae biochar production: A renewed possibility. *Bioresource Technology*, *388*, 129748. https://doi.org/10.1016/j.biortech.2023.129748

Wyder, A. B., Boss, R., Naskova, J., Kaufmann, T., Steiner, A., & Graber, H. U. (2011). *Streptococcus* spp. and related bacteria: Their identification and their pathogenic potential for chronic mastitis – A molecular approach. *Research in Veterinary Science*, *91*(3), 349–357. https://doi.org/10.1016/j.rvsc.2010.09.006

Xin, H., Gao, M., Wang, X., Qiu, T., Guo, Y., & Zhang, L. (2022). Animal farms are hot spots for airborne antimicrobial resistance. *Science of The Total Environment*, *851*, 158050. https://doi.org/10.1016/j.scitotenv.2022.158050

Yaici, L., Haenni, M., Saras, E., Boudehouche, W., Touati, A., & Madec, J.-Y. (2016). *Bla* $_{NDM-5}$ -carrying IncX3 plasmid in *Escherichia coli* ST1284 isolated from raw milk collected in a dairy farm in Algeria. *Journal of Antimicrobial Chemotherapy*, *71*(9), 2671–2672. https://doi.org/10.1093/jac/dkw160

Yamagishi, J., Sato, Y., Shinozaki, N., Ye, B., Tsuboi, A., Nagasaki, M., & Yamashita, R. (2016). Comparison of Boiling and Robotics Automation Method in DNA Extraction for Metagenomic Sequencing of Human Oral Microbes. *PLoS ONE*, *11*(4), e0154389. https://doi.org/10.1371/journal.pone.0154389

Yan, H., Liu, C., Yu, W., Zhu, X., & Chen, B. (2023). The aggregate distribution of *Pseudomonas aeruginosa* on biochar facilitates quorum sensing and biofilm formation. *Science of The Total Environment*, *856*, 159034. https://doi.org/10.1016/j.scitotenv.2022.159034

Yan, X., Ying, Y., Li, K., Zhang, Q., & Wang, K. (2024). A review of mitigation technologies and management strategies for greenhouse gas and air pollutant emissions in livestock production. *Journal of Environmental Management*, *352*, 120028. https://doi.org/10.1016/j.jenvman.2024.120028

Yang, Z., Yu, Y., Hu, R., Xu, X., Xian, J., Yang, Y., Liu, L., & Cheng, Z. (2020). Effect of rice straw and swine manure biochar on N_2O emission from paddy soil. *Scientific Reports*, *10*(1), 10843. https://doi.org/10.1038/s41598-020-67705-z

Yong Ng, L. S., Tan, T. Y., & San Yeow, S. C. (2010). A cost-effective method for the presumptive identification of Enterobacteriaceae for diagnostic microbiology laboratories. *Pathology*, *42*(3), 280–283. https://doi.org/10.3109/00313021003631338

Yu, Z. N., Wang, J., Ho, H., Wang, Y. T., Huang, S. N., & Han, R. W. (2020). Prevalence and antimicrobial-resistance phenotypes and genotypes of Escherichia coli isolated from raw milk samples from mastitis cases in four regions of China. *Journal of Global Antimicrobial Resistance*, *22*, 94–101. https://doi.org/10.1016/j.jgar.2019.12.016

Zaidi, S.-Z., Zaheer, R., Barbieri, R., Cook, S. R., Hannon, S. J., Booker, C. W., Church, D., Van Domselaar, G., Zovoilis, A., & McAllister, T. A. (2022). Genomic

Characterization of *Enterococcus hirae* From Beef Cattle Feedlots and Associated Environmental Continuum. *Frontiers in Microbiology*, *13*. https://www.frontiersin.org/articles/10.3389/fmicb.2022.859990

Zalewska, M., Błażejewska, A., Czapko, A., & Popowska, M. (2021). Antibiotics and Antibiotic Resistance Genes in Animal Manure – Consequences of Its Application in Agriculture. *Frontiers in Microbiology*, *12*, 610656. https://doi.org/10.3389/fmicb.2021.610656

Zdanowicz, M., Shelford, J. A., Tucker, C. B., Weary, D. M., & von Keyserlingk, M. A. G. (2004). Bacterial Populations on Teat Ends of Dairy Cows Housed in Free Stalls and Bedded with Either Sand or Sawdust. *Journal of Dairy Science*, *87*(6), 1694–1701. https://doi.org/10.3168/jds.S0022-0302(04)73322-6

Zhang, H., Voroney, R. P., & Price, G. W. (2014). Effects of Biochar Amendments on Soil Microbial Biomass and Activity. *Journal of Environmental Quality*, *43*(6), 2104–2114. https://doi.org/10.2134/jeq2014.03.0132

Zhang, J., Ge, X., Qiu, X., Liu, L., Mulder, J., & Duan, L. (2024). Estimation of carbon sequestration potential and air quality impacts of biochar production from straw in China. *Environmental Pollution (Barking, Essex: 1987)*, *363*(Pt 2), 125304. https://doi.org/10.1016/j.envpol.2024.125304

Zhang, M., Xie, W., Zhong, X., Wang, Y., Li, S., Zhou, Y., & Wang, C. (2024). The impact of combined application of biochar and fertilizer on the biochemical properties of soil in soybean fields. *PeerJ*, *12*, e18172. https://doi.org/10.7717/peerj.18172

Zhang, X., Yi, X., Zhuang, H., Deng, Z., & Ma, C. (2022). Invited Review: Antimicrobial Use and Antimicrobial Resistance in Pathogens Associated with Diarrhea and Pneumonia in Dairy Calves. *Animals*, *12*(6), 771. https://doi.org/10.3390/ani12060771

Zhang, X., Zhen, D., Liu, F., Chen, R., Peng, Q., & Wang, Z. (2023). An achieved strategy for magnetic biochar for removal of tetracyclines and fluoroquinolones: Adsorption and mechanism studies. *Bioresource Technology*, *369*, 128440. https://doi.org/10.1016/j.biortech.2022.128440

Zhang, Y., Hao, X., Thomas, B. W., McAllister, T. A., Workentine, M., Jin, L., Shi, X., & Alexander, T. W. (2023). Soil antibiotic resistance genes accumulate at different rates over four decades of manure application. *Journal of Hazardous Materials*, *443*, 130136. https://doi.org/10.1016/j.jhazmat.2022.130136

Zhou, K., Sun, L., Zhang, X., Xu, X., Mi, K., Ma, W., Zhang, L., & Huang, L. (2023). Salmonella antimicrobials inherited and the non-inherited resistance: Mechanisms and alternative therapeutic strategies. *Frontiers in Microbiology*, *14*. https://www.frontiersin.org/articles/10.3389/fmicb.2023.1176317

Zhu, T., Su, Z., Lai, W., Zhang, Y., & Liu, Y. (2021). Insights into the fate and removal of antibiotics and antibiotic resistance genes using biological wastewater treatment

technology. *Science of The Total Environment*, *776*, 145906. https://doi.org/10.1016/j.scitotenv.2021.145906