PhD Thesis
Kevin S. Jerez Bogota

AARHUS
UNIVERSITY

Department of
Food Science

Could combining plants be the key to enhancing livestock health and reducing antibiotic use? This question prompted this thesis's research into the enhanced antibacterial properties of plant combinations to control gastrointestinal infections in piglets and chickens. In the study with organically raised piglets, combinations of garlic and fruit powders were used to mitigate pathogenic *E. coli* infections, effectively preventing postweaning diarrhea and promoting a healthier gut microbiome. Research on chickens examined the efficacy of combining a natural source of antibacterial fatty acids and essential oils to combat necrotic enteritis caused by pathogenic *C. perfringens*, reducing the disease's impact.

The PhD research project was conducted as part of MonoGutHealth, a training network funded by the European Commission (Marie Skłodowska-Curie Actions).

Multicomponent Antibacterial Plant Cocktail for Better Health in Piglets and Broilers

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ANTIBACTERIAL PLANT COMBINATIONS FOR BETTER GASTROINTESTINAL HEALTH IN PIGLETS AND BROILERS

KEVIN S. JEREZ BOGOTA

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Antibacterial Plant Combinations for Better gastrointestinal Health in Piglets and Broilers

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Differentiated & Biofunctional Foods

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AARHUS
UNIVERSITY
DEPARTMENT OF FOOD SCIENCE

"Por fortuna, Macondo no es un lugar, sino un estado de ánimo"

-Gabriel García Márquez

"*It is important to draw wisdom from many different places. If we take it from only one place, it becomes rigid and stale. Understanding others, the other elements, and the other nations will help you become whole.*"

- Iroh.

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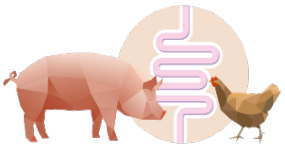
PREFACE

The PhD project "Multicomponent Antibacterial Plant Cocktail for Better Health in Piglets and Broilers" was done in connection to the MonoGutHealth (Training and research for sustainable solutions to support and sustain gut health and reduce losses in monogastric livestock) project, an Innovative Training Network funded by the European Commission under the Horizon 2020 Marie Skłodowska-Curie Action. Additionally, part of the experimental work contributed to the MAFFRA II project (Plant Cocktails with Antibacterial Effect Against Weaning Diarrhea in Organic Piglets—Applied Proof of Concept) funded by the ORDD4 initiative from the Green Development and Demonstration Program (GUDP) under the Danish Ministry of Environment and Food, coordinated by ICROFS (International Centre for Research in Organic Food Systems).

The experimental work was conducted at Aarhus University, Denmark in the Departments of Animal and Veterinary Science, and Food Science. Part of the 16S rRNA sequencing data analysis was done during a three-month research stay at Teagasc Morepark in Ireland under the guidance of Gillian Gardiner, Peadar Lawlor, and Paul Cormican. Furthermore, two half-month secondment placements were completed with the Center for Free-Range Animals (*Centre for Frilandsdyr*) and DLG in Denmark, where training was provided on sustainable feed practices, commercial production, and the use of feed additives in pig farming.



monogut health

Optimal gut function in monogastric livestock

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ICROFS
International Centre for Research in Organic Food Systems

Ministeriet for Fødevarer, Landbrug og Fiskeri
gudp

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I am grateful for having the chance to be part of Aarhus University and the Department of Food Science, a greatly diverse and dynamic academic community. Furthermore, I greatly value my participation in the MonoGutHealth-Marie Curie training network. It has been a unique opportunity to be part of a large network of researchers, industry partners, communication experts, and colleagues. I am confident that the impact of this initiative will accompany me for many years. To my fellow "ESRs" Daria, Inés, Modou, Muhammad, John, Ramesha, Roberta, Shiv, Tobias, Wendy, and Pauline. Thanks to Giuseppe Bee and all the advisors for their inspiring leadership and for pushing us to be the best versions of ourselves. I will greatly miss the project meetings.

I would like to acknowledge the involvement and support of colleagues and collaborators: Anna Wessels and particularly Kathrin Werth from Kaesler Nutrition; Rikke Thomsen and the team at CFF; Theresa Nyborg Carl and the nutrition team at DLG; Paul Cormican, Gillian Gardiner, and Peadar Lawlor from Teagasc, Ireland. Their valuable input, constructive criticism, and shared knowledge have contributed to the work presented here. Thanks to Trinne Poulsen for her immense dedication and experience. Thanks to Lene Rosborg Dal, Kasper Vrangstrup Poulsen, Thomas Rebsdorf, and Inger Marie Jepsen for their patience and expertise. Also, words of gratitude to Tina Sørensen, Rikke Brødsgaard Kjærup, Anna Schönherz, Samantha Noel, Dar'ya Vodolazs'ka, Ruben Grosso, Élisabeth Chassé, Gavin Simpson, Leslie Foldager, and Mihai Curtasu for the fruitful discussions and advice. To all the GHH group at AU-ANIVET, thank you for being so kind to this "never-leaving" visitor. Thank you for your support, encouragement, and for making the research environment both productive and enjoyable. To my mentor, Tofuko Woyengo, thank you for "showing me the way". If someone should be mentioned twice to show how much I appreciate her support, that should be Nuria Canibe, i*nfinitas gracias!*

I would also like to extend my gratitude to my PhD colleagues. Especially, Jiajia Xu, Despoina Georgaki, Li-Hsuan Chen, and Pernille Aagaard Madsen, for being my family in Denmark. Your camaraderie, collaboration, and insightful discussions have been invaluable. The shared experiences, challenges, and triumphs have made this journey memorable and rewarding. To the PhD students at Teagasc in Ireland for hosting me during one of the most memorable three months of my life. Thanks to Roberta, and my "frienchs" Elisa, Mathew, Allan, Laeticia, and Florance. *Merci!.* To my friends and family, the "distance," thank you all for your constant support, understanding, and for bringing joy to my life; you are always on my mind. Adriana Alvarez, thank you for "saving my life" in so many ways. To Ivette Manjarrez, Jimena Ibagon, and Ricardo Garavito, thank you for making me your family for many years (and so many more to come); thank you for the laughs, the arguments, the encouragement, and for letting me be a part of your souls (and for being part of mine). Can't wait to see us when *I'm sixty-four* . My heart is with my family: my mother, Luz Bogota, my greatest inspiration and moral cornerstone; my father, Efrain Jerez, for his unconditional support; and my brothers, Johs Jerez and Michael Sarmiento, thank you for enriching my mind with your thoughts and being there for me. To my extended family in Colombia, thank you for having me in your thoughts.

SUMMARY

With growing efforts to address antimicrobial resistance, environmental pollution, and sustainable development, there is a substantial drive to reduce conventional antimicrobials in food systems. Plant-based antibacterial combinations offer a promising solution to control pathogens and support animal health. Piglets and broilers are prone to enteric diseases due to production conditions and physiological stress. Weaning stress leads to Enterotoxigenic *Escherichia coli* (**ETEC**) proliferation in piglets, causing postweaning diarrhea (**PWD**). Similarly, *netB*+ *Clostridium perfringens* in broilers leads to necrotic enteritis. Both diseases impose significant economic burdens, highlighting the need for sustainable solutions. This PhD thesis investigates plant-based antimicrobials to control ETEC-induced PWD in piglets and *netB*+ *C. perfringens*-induced necrotic enteritis in broilers. The aim is to innovate a plant-based antibacterial combination approach to prevent gastrointestinal bacterial diseases in farm animals, reducing reliance on conventional antimicrobials.

Study I investigated the efficacy of dietary supplementation of garlic combined with apple pomace or blackcurrant against PWD induced by ETEC in organically raised piglets. Piglets receiving these treatments had better growth performance, reduced diarrhea, and reduced ETEC excretion compared to those that did not receive the supplements. The combinations also positively impacted the diversity and stability of the fecal microbiota, indicating that these supplements can selectively target gut pathogens and support piglet health. **Study II** further evaluated the impacts of dietary supplementation of garlic combined with apple pomace or blackcurrant on the gastrointestinal ecosystem of pigs fed organic diets three weeks after ETEC challenge and treatment supplementation. Results showed that while ETEC-challenged pigs had a higher abundance of potential pathogens, pigs fed the supplemented diets had higher abundance of potentially beneficial bacteria and improved fermentation profiles, favoring carbohydrate over protein fermentation. Overall, garlic combined with apple pomace or blackcurrant promoted a healthier gut microbial ecosystem. **Study III** investigated the efficacy of dietary supplementation with palm kernel fatty acids (rich in lauric acid) and clove essential oil against necrotic enteritis induced by *netB*+ *C. perfringens* in broiler chickens. In vitro testing revealed synergistic antibacterial activity of the combination. The chickens receiving the combination showed improved growth performance, reduced intestinal lesions, and lower *C. perfringens* counts compared to the control group. The combination primarily modulated the gut microbiota during the infection, but lower *C. perfringens* abundance was maintained over time. Overall, the combination of palm kernel fatty acids and clove essential oil effectively controlled necrotic enteritis.

This thesis underscores the effectiveness of plant-based antibacterial combinations in controlling gastrointestinal pathogens. The studies showed that the combinations reduced disease severity and improved animal performance by selectively targeting pathogens without negatively impacting the gut microbial ecosystem. These solutions offer a valuable tool for managing enteric diseases, reducing dependency on conventional antimicrobials, and supporting sustainable practices. Future studies should refine the antibacterial concentrations of plant combinations, investigate more complex or different combinations, narrow down active components and their synergies, explore complementarity with other strategies, and assess their practical effectiveness and cost-benefit scenarios under different settings.

SAMMENDRAG

Med en voksende indsats for at adressere antimikrobiel resistens, miljøforurening og bæredygtig udvikling er der en betydelig motivation for at reducere konventionelle antimikrobielle stoffer i fødevaresystemer. Plantebaserede antibakterielle kombinationer er en lovende løsning til at kontrollere patogener og understøtte dyrenes sundhed. Smågrise og slagtekyllinger er tilbøjelige til at få tarmsygdomme på grund af produktionsforhold og fysiologisk stress. Fravænningsstress fører til proliferation af Enterotoksigene *Escherichia coli* (**ETEC**) hos smågrise, hvilket forårsager fravænningsdiarré (**PWD**). Tilsvarende fører *netB*+ *Clostridium perfringens* hos slagtekyllinger til nekrotisk enteritis. Begge sygdomme medfører betydelige økonomiske byrder, hvilket understreger behovet for bæredygtige løsninger. Denne ph.d.-afhandling undersøger plantebaserede antimikrobielle stoffer til kontrollering af ETEC-induceret PWD hos smågrise og *netB*+ *C. perfringens*-induceret nekrotisk enteritis hos slagtekyllinger. Formålet er at innovere en plantebaseret kombinationstilgang til forebyggelse af gastrointestinale bakteriesygdomme hos husdyr og dermed mindske afhængigheden af konventionelle antimikrobielle stoffer.

Forsøg I undersøgte effekten af et fodersupplement med hvidløg kombineret med æble pomace eller solbær mod PWD induceret af ETEC hos økologisk opdrættede smågrise. Smågrise, der modtog disse behandlinger, havde bedre vækstydelse, reduceret diarré og nedsat ETEC-udskillelse sammenlignet med dem, der ikke modtog et fodersupplement. Kombinationerne påvirkede også positivt diversiteten og stabiliteten af den fækale mikroflora, hvilket indikerer, at disse fodersupplementer selektivt kan angribe tarmpatogener og understøtte smågrises sundhed. **Forsøg II** evaluerede yderligere effekten af fodersupplementet med hvidløg kombineret med æble pomace eller solbær på det gastrointestinale økosystem hos svin, der fodres med økologisk foder tre uger efter ETEC-challenge og tildeling af foderbehandling. Resultaterne viste, at mens ETEC-challenged grise havde en højere andel af potentielle patogener, havde de grise der fik tildelt fodersupplement en højere andel af potentielt gavnlige bakterier og forbedrede fermenteringsprofiler, hvilket favoriserede kulhydrat- frem for proteinfermentering. Samlet set fremmede hvidløg kombineret med æble pomace eller solbær et sundere tarmmikrobielt økosystem. **Forsøg III** undersøgte effekten af et fodersupplement med palmekernefedtsyrer (rig på laurinsyre) og æterisk kløverolie mod nekrotisk enteritis induceret af *netB*+ *C. perfringens* hos slagtekyllinger. In vitro-test afslørede forstærket antibakteriel aktivitet på baggrund af kombinationen. Kyllingerne, der modtog kombinationen, viste forbedret vækstydelse, reducerede tarmlæsioner og lavere *C. perfringens* forekomst sammenlignet med kontrolgruppen. Kombinationen modulerede primært tarmmikrobiotaen under infektionen, men lavere forekomst af *C. perfringens* blev opretholdt over tid. Samlet set kontrollerede kombinationen af palmekernefedtsyrer og æterisk kløverolie effektivt nekrotisk enteritis.

Denne afhandling understreger effektiviteten af plantebaserede antibakterielle kombinationer til kontrollering af gastrointestinale patogener. Forsøgene viste, at kombinationerne reducerede sværhedsgraden af sygdommene og forbedrede dyrenes ydeevne ved selektivt at angribe patogener uden at påvirke tarmens mikrobielle økosystem negativt. Disse løsninger udgør et værdifuldt redskab til håndtering af enteriske sygdomme, reduktion af afhængigheden af konventionelle antimikrobielle stoffer og understøtter bæredygtig praksis. Fremtidige forsøg bør raffinere den antimikrobielle koncentration af plantekombinationerne, indskrænke aktive stoffer og deres synergieffekter, udforske komplementaritet med andre strategier og vurdere deres praktiske effektivitet og nytteværdi scenarier under forskellige forhold.

RESUMEN

Con los crecientes esfuerzos para abordar la resistencia a los antimicrobianos, la contaminación ambiental y el desarrollo sostenible, existe un impulso sustancial para reducir los antimicrobianos convencionales en los sistemas alimentarios. Las combinaciones antibacterianas de origen vegetal ofrecen una solución prometedora para controlar patógenos y promover la salud animal. Los lechones y los pollos de engorde son propensos a enfermedades entéricas debido a las condiciones de producción y el estrés fisiológico. El estrés del destete conduce a la proliferación de *Escherichia coli* enterotoxigénica (ETEC) en los lechones, causando diarrea post-destete (DPD). De manera similar, *Clostridium perfringens* netB+ en los pollos de engorde provoca enteritis necrótica. Ambas enfermedades imponen cargas económicas significativas, destacando la necesidad de soluciones sostenibles. Esta tesis doctoral investiga los antimicrobianos de origen vegetal para controlar la DPD inducida por ETEC en lechones y la enteritis necrótica inducida por *C. perfringens* netB+ en pollos de engorde. El objetivo es innovar un enfoque de combinación antibacteriana de origen vegetal para prevenir enfermedades bacterianas gastrointestinales en animales de granja, reduciendo la dependencia de los antimicrobianos convencionales.

El Estudio I investigó la eficacia de la suplementación dietética de ajo combinado con orujo de manzana o grosella negra contra la DPD inducida por ETEC en lechones criados orgánicamente. Los lechones que recibieron estos tratamientos tuvieron un mejor rendimiento, menos diarrea y menor excreción de ETEC en comparación con aquellos que no recibieron los suplementos. Las combinaciones también impactaron positivamente en la diversidad y estabilidad de la microbiota fecal, indicando que estos suplementos pueden atacar selectivamente a los patógenos intestinales y apoyar la salud de los lechones. El Estudio II evaluó además los impactos de la suplementación de ajo combinado con orujo de manzana o grosella negra en el ecosistema gastrointestinal de cerdos alimentados con dietas orgánicas tres semanas después del desafío con ETEC y la suplementación del tratamiento. Los resultados mostraron que, mientras los cerdos desafiados con ETEC tenían una mayor abundancia de patógenos potenciales, los cerdos suplementados tenían una mayor abundancia de bacterias potencialmente beneficiosas y perfiles de fermentación mejorados, favoreciendo la fermentación de carbohidratos sobre la de proteínas. En general, el ajo combinado con orujo de manzana o grosella negra promovió un ecosistema microbiano intestinal más saludable. El Estudio III investigó la eficacia de la suplementación dietética con ácidos grasos de núcleo de palma (ricos en ácido láurico) y aceite esencial de clavo contra la enteritis necrótica inducida por *C. perfringens* netB+ en pollos de engorde. Las pruebas in vitro revelaron una actividad antibacteriana sinérgica de la combinación. Los pollos que recibieron la combinación mostraron un mejor rendimiento de crecimiento, menos lesiones intestinales y menores conteos de *C. perfringens* en comparación con el grupo de control. La combinación moduló principalmente la microbiota intestinal durante la infección, pero la menor abundancia de *C. perfringens* se mantuvo con el tiempo. En general, la combinación de ácidos grasos de núcleo de palma y aceite esencial de clavo controló eficazmente la enteritis necrótica. Esta tesis subraya la efectividad de las combinaciones antibacterianas de origen vegetal en el control de patógenos gastrointestinales. Los estudios mostraron que las combinaciones redujeron la severidad de la enfermedad y mejoraron el rendimiento animal al atacar selectivamente a los patógenos sin impactar negativamente el ecosistema microbiano intestinal. Estas soluciones ofrecen una herramienta valiosa para manejar las enfermedades entéricas, reducir la dependencia de los antimicrobianos convencionales y apoyar prácticas sostenibles. Los estudios futuros deberían refinar las concentraciones antibacterianas de las combinaciones vegetales, investigar combinaciones más complejas o diferentes, identificar los componentes activos y sus sinergias, explorar la complementariedad con otras estrategias y evaluar su efectividad práctica y escenarios de costo-beneficio en diferentes entornos.

LIST OF INCLUDED MANUSCRIPTS

Study I:

Antibacterial plant combinations prevent postweaning diarrhea in organically raised piglets challenged with enterotoxigenic *Escherichia coli* F18

Jerez-Bogota K, Jensen M, Højberg O, Cormican P, Lawlor PG, Gardiner GE and Canibe N

Published: Frontiers in Veterinary Science, 10. DOI: 10.3389/fvets.2023.1095160

Study II:

Effects of Garlic with Apple Pomace or Blackcurrant Supplementation on the Gastrointestinal Microbial Ecosystem in Organic Pigs After Weaning

Kevin Jerez-Bogota, Martin Jensen, Ole Højberg and Nuria Canibe

Manuscript ready for submission to: Journal of Animal Science.

Study III:

Efficacy of Medium Chain Fatty Acids and Essential Oils Against Necrotic Enteritis in Broilers

Kevin Jerez-Bogota, Martin Jensen, Ole Højberg, Nuria Canibe and Ricarda Engberg

Manuscript ready for submission to: Poultry Science.

LIST OF OTHER CONTRIBUTIONS DURING THE PHD

Feeding antibacterial plant combinations to mitigate post-weaning diarrhoea in organic piglets challenged with enterotoxigenic *Escherichia coli* F18. Jerez-Bogota, K. S., Hojberg, O., Jensen, M. & Canibe, N., Jul 2022, Digestive Physiology of Pigs 2022 - Rotterdam, Netherlands. Poster. In: Animal - Science proceedings. 13, 2, p. 218 1 p., P195.

Plantecocktail reducerer colibakterier hos øko-smågrise. Jensen, M., Jerez-Bogota, K. S., Hojberg, O. & Canibe, N., Mar 2022, In: Økologi - Inspiration til jordbruget. 2. Contribution to newspaper, Communication.

Plantecocktail reducerer colibakterier hos øko-smågrise. Jerez-Bogota, K. S., Hojberg, O., Jensen, M. & Canibe, N., Jul 2022, Aarhus Universitet - DCA - Nationalt Center for Fødevarer og Jordbrug. Communication

Plantecocktail reducerer diarré hos økologiske fravænningsgrise. Jerez-Bogota, K. S., Hojberg, O., Jensen, M. & Canibe, N., Jun 2022, In: Hyologisk. p. 32-35. Contribution to newspaper, Communication.

Antibacterial plant blends modulate gut microbiota in organic piglets challenged with *E. coli* F18. K. Jerez-Bogota, M. Jensen, O. Højberg and N. Canibe. Book of Abstracts of the 74th Annual Meeting of the European Federation of Animal Science. Published: 2023 Pages: 1092. DOI:10.3920/978-90-8686-936-7

MonoGutHealth D2.11 Microbiome composition and plant additives. Jerez-Bogota K, Jensen M, Højberg O, Cormican P, Lawlor PG, Gardiner GE and Canibe N. Project deliverable report.

MonoGutHealth D3.6 Antibacterial plant stability and efficacy in vivo. Jerez-Bogota K, Jensen M, Højberg O, and Canibe N. Project deliverable report.

MonoGutHealth D3.2 Antibacterial plant stability and efficacy in vitro. Jerez-Bogota K, Jensen M, Canibe N, Engberg R, Højberg O. Project deliverable report.

MonoGutHealth General Project Meetings Presentations: Poland, Germany, Belgium and Ireland (Best presenter award).

MY OWN CONTRIBUTION TO THE THESIS

The thesis document was written by me and received advise and feedback from my supervisors, primarily Martin Jensen and Nuria Canibe. Emma Bonde Stanek proofread the summary and translated it into Danish (Sammendrag). Pernille Aagaard Madsen reviewed the Sammendrag.

Study I: The experimental conception and desing was conceived by my advisors before I joined the project. The animal experiment and sampling were conducted by researchers and technicians at the Department of Animal and Veterinary Science Aarhus University (AU-ANIVET) before I arrived in Denmark. Although I was anticipated to participate, the delay in my arrival due to the COVID pandemic prevented my involvement. I performed DNA extraction, qPCR enumerations assay and prepared samples for DNA sequencing with guidance from Trine Poulsen (AU-ANIVET), with sequencing data procured by Samantha Joan Noel (AU-ANIVET). I coordinated the analysis of feed samples, blood acute phase proteins, the analyses were carried out by technicians (AU-ANIVET). Allicin content in garlic were done at the department of Food Science of Aarhus University (AU-FOOD), coordinated by Martin Jensen. I performed calculations and statistical analysis, receiving advice from Leslie Foldager, Gavin Simpson and Nuria Canibe (AU-ANIVET). During a research stay at Teagasc, Ireland, I received bioinformatics teaching from Paul Cormican, I completed the analyses and received feedback from Anna Schönherz (AU-ANIVET). I interpreted the data and wrote the manuscript, incorporating ideas, revisions and feedback from coauthors.

Study II: The study used samples taken from animal experiments in Study I. The analysis of microbial metabolites in the digesta was performed by me with guidance from Thomas Rebsdorf (AU-ANIVET). The mucosal gene expression and antioxidant enzyme activities protocols and analyses were developed by me with advice from Kasper Poulsen, Inger Marie Jepsen, Dar'ya Vodolazs'ka and Rikke Brødsgaard Kjærup (AU-ANIVET). I performed DNA extraction, qPCR enumerations assay and prepared samples for DNA sequencing with guidance from Trine Poulsen, with sequencing data procured by Samantha Joan Noel. I performed calculations, bioinformatics and statistical analysis receiving advice from Nuria Canibe and Anna Schönherz. I interpreted the data and wrote the manuscript, incorporating ideas, revisions and feedback from coauthors.

Study III: The study was conceived by me and my advisors. Nuria Canibe and I coordinated the procurement of oils. I designed the in vitro experiment with guidance from Ole Højberg and assistance from Trine Poulsen. Ricarda Margarete Engberg and I developed the experimental design and animal experiment, which was conducted at AU-VIBORG with the help of the animal facilities staff. Trine Poulsen and I handled sampling preparation and logistics. The challenge protocol was performed by me, Ricarda Margarete Engberg, and Trine Poulsen. Blood and gene expression samples and analyses (part of Konstantinos Papanikolaou's Master Thesis project) were carried out by Lene Rosborg Dal and Rikke Brødsgaard Kjærup. I co-supervised Konstantinos Papanikolaou, providing insights into jejunal tissue gene expression and statistical analyses. I performed DNA extraction and prepared samples for DNA sequencing with guidance from Trine Poulsen, with sequencing data procured by Anna Schönherz. Trine Poulsen conducted the qPCR enumerations assays. I performed calculations, bioinformatics, and statistical analysis with advice from Nuria Canibe. I interpreted the data and wrote the manuscript, incorporating ideas, revisions, and feedback from coauthors.

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LIST OF ABBREVIATIONS

ADG	Average Daily Gain
ADFI	Average Daily Feed
ANE	Avian Necrotic Enteritis
BW	Body Weight
cDNA	Complementary DNA
CFU	Colony-Forming Units
CP	Crude Protein
DAS	Diallyl Sulfide
DADS	Diallyl Disulfide
DATS	Diallyl Trisulfide
DM	Dry Matter
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Antibody Assay
ETEC	Enterotoxigenic Escherichia coli
HPLC	High-Performance (High-Pressure) Liquid Chromatography
Ig	Immunoglobulin
IL	Interleukin
IU	International Unit
LPS	Lipopolysaccharide
MCFA	Medium Chain Fatty Acid
MDR	Multidrug Resistant
MIC	Minimum Inhibitory Concentrations
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
PCA	Principal Component Analysis
PKFA	Palm Kernel Fatty Acids
PCR	Polymerase Chain Reaction
Pig-MAP	Pig Major Acute Phase Protein
PWD	Postweaning Diarrhea
qPCR	Quantitative PCR
RNA	Ribonucleic Acid
SCFA	Short Chain Fatty Acid

GENERAL INTRODUCTION

"*...in the last years the modern western world has been learning what many Asians and native Americans have known for centuries, namely that plant extracts and spices can play a significant role in health and nutrition*" (Wenk, 2003).

Chicken and pig meat have become the dominant types of meat produced globally, accounting for approximately 40% and 35% of total global production, respectively (FAO STAT, 2023). The trend is primarily driven by technological advancements and improved efficiency. Additionally, societal demand for affordable, safer, and more sustainable products has favored the pig and poultry industries, which are noted for their technological progress compared to other livestock systems.

The livestock industry as a whole is moving towards more sustainable and health-conscious production methods (Hongguang, 2023). Paradoxically, increased sustainability and efficiency often lead to higher intensification. Researchers agree that modern livestock husbandry practices have put significant pressure on biological systems, resulting in increased stress on both the systems and the animals (Soren, 2012). One prominent consequence is a heightened susceptibility to infectious diseases (Duarte et al., 2023b). Furthermore, there has been significant attention on gastrointestinal health issues in monogastric animals, partly due to the resurgence of enteric diseases following the reduction of routine antibiotic use in livestock systems (Patience and Ramirez, 2022) and restrictions on zinc oxide due to environmental concerns (EMA and EFSA, 2017).

Both piglets and broilers are prone to enteric diseases when the production conditions and their physiological state allow pathogens to thrive. In pig production, weaning is a critical period marked by physiological and environmental stressors, contributing to the onset of postweaning diarrhea (Gresse et al., 2017). Pathogenic strains of *Escherichia coli* are the main infectious agents behind postweaning diarrhea in piglets (Francis, 2002). In broiler chickens, nutritional, parasitic, and environmental stress between 2-6 weeks post-hatch can lead to necrotic enteritis, characterized by diarrhea and intestinal mucosal necrosis (Dahiya et al., 2006). The primary culprit here is the overgrowth of *Clostridium perfringens* in the small intestine (Abildgaard et al., 2010b). Both diseases impose significant economic burdens on pig and poultry production, and their resurgence following antibiotic bans underscores the need for new methods to support and enhance gut health in young monogastrics.

In addition to addressing pathogenic bacteria, understanding the complex interplay between gut microbiota and the host is crucial (Forgie et al., 2019). Alterations in the gut microbiome can significantly impact nutrient absorption, immune response, and disease resistance (Patil et al., 2020). A holistic understanding of these interactions is essential for developing sustainable strategies to support animal health. In this context, the MonoGutHealth project aims to investigate novel strategies in livestock health, focusing on early-life microbiome development in the gastrointestinal tract as an alternative to traditional antibiotics, thereby enhancing sustainability in pig and poultry production.

One promising alternative is the use of botanicals—plants or plant extracts with therapeutic properties, such as herbs, spices, and extracts (also known as phytogenics or phytobiotics). This PhD project focuses on developing multicomponent antibacterial plant combinations to

improve health in piglets and broilers. The aim is to innovate a plant-based approach to prevent bacterial diseases in farm animals, potentially reducing reliance on antibiotics, heavy metals, and coccidiostats.

The work presented in this thesis builds upon the contribution of previous projects in conjunction with parallel initiatives— MAFFRA I (From January 1st, 2016, to December 31st, 2017), MAFFRA II (January 1st, 2019, to October 31st, 2022), and Monoguthealth (From June 1st, 2021, to present).

During MAFFRA I (Antibacterial Plants Against Diarrhea in Pig Herds), twelve plant species were identified for their activity against pathogenic *E. coli*. Ramsons (wild garlic) and acidic berries were the most effective in vitro, demonstrating both antibacterial and acidifying properties. In a pilot study, weaning pigs were fed a mixture of ramsons and lingonberries, which showed a significant reduction in *E. coli* levels without affecting Lactobacillus populations or feed intake. MAFFRA II (Plants as Antibacterial Feed for Preventing Diarrhea in Piglets), aimed to refine and scale the findings from the pilot study (MAFFRA I) using more readily available raw materials and food production "side streams". The activities included in vitro testing of garlic, apple pomace, and blackcurrant against ETEC, the in vivo challenge experiment documented in Study I of this thesis, and a demonstration test in a commercial organic pig farm using garlic and apple pomace (conducted by CFF).

The Monoguthealth (Optimal Gut Function in Monogastric Livestock) project provided funding for the PhD program as part of the Marie Skłodowska-Curie scholarship, as well as funding for the experimental work conducted in Study II and Study III of the current thesis. Additionally, Monoguthealth provided the structure for the PhD program as part of the Innovative Training Network (11 PhD students across 6 institutions, 24 academic and industrial partners) courses, training and dissemination activities.

Thesis Outline

Chapter 1: BACKGROUND	Provides an overview of the etiology and pathophysiology of these diseases, describe specific antibacterial plant materials of interest, and analyzes their potential for improving antibacterial efficacy through synergistic combinations.
Chapter 2: METHODOLOGY	Present an overview of the methods for assessing their antibacterial plants efficacy both in vitro and in vivo, and measurements of their effects on gut microbiota and host responses with particular focus on pig PWD and chicken ANE.
Chapter 3: STUDY OVERVIEW	A summary of the studies desing and results and an assessment of the applied methodologies
Chapter 4: GENERAL DISCUSSION	Provides a general discussion of the results in the thesis with reference to the general literature
Chapter 5: CONCLUSSION AND PERSPECTIVES	Provides a general discussion of the results in the thesis with reference to the general literature
Study I, II and III	Attached manuscripts

Chapter 1: BACKGROUND

1.1 INTRODUCTION

Young monogastrics are vulnerable to pathogenic bacteria, which can result in gastrointestinal disorders and significant economic losses. The weaning process in piglets, characterized by environmental and dietary changes, often paves the way for the onset of postweaning diarrhea (**PWD**). Meanwhile, in broiler chickens, similar stressors around 2-4 weeks of life can trigger the onset of avian necrotic enteritis (**ANE**). In both cases, the etiological agent is a pathobiont[1], Enterotoxigenic *Escherichia coli* (**ETEC**) and *Clostridium perfringens* type G are the bacteria involved in PWD and ANE, respectively. Antibiotics have been used for controlling PWD and ANE but concerns over antimicrobial resistance (**AMR**) led to restrictions[2]. Furthermore, supra-nutritional levels of zinc oxide presented a solution for PWD, but its use in the EU has also recently been restricted over environmental pollution concerns. The broiler industry, on the other hand, currently relies on the use of ionophore coccidiostats for control of ANE. Notwithstanding, "antibiotic free" or "non-antibiotics ever" schemes have been promoted by societal views over antibiotic usage, encouraging processors and retailers (e.g., meat packing companies, supermarket chains and food companies) to demand or economically incentivize these concepts for meat production.

The search for alternative strategies to conventional antimicrobials in livestock systems is a highly relevant and dynamic research area. Several approaches have been explored, including management and infrastructure innovations, improved hygiene and biosecurity, breeding programs, and nutritional strategies (Patience and Ramirez, 2022). Nutritional strategies are being thoroughly investigated due to their significant impact on productivity. Optimizing nutrient requirements for young monogastrics and adding functional substances to feed are the most studied nutritional approaches (Lallès et al., 2009). Interest has surged in plants and plant-based bioactive substances as effective, sustainable, and consumer-acceptable alternatives to conventional antimicrobials. Indeed, a substantial amount of research has focused on various plants and plant-derived substances as alternatives to antibiotics (Patience and Ramirez, 2022, Abd El-Hack et al., 2022).

Among these alternatives, botanicals, phytogenics, phytobiotics, and phytochemicals have been investigated as promising candidates (see box below). Phytochemicals, such as flavonoids (quercetin, apigenin), terpenes (limonene, pinene), organosulfurs (allicin, isothiocyanates), and phenolic acids (caffeic acid, gallic acid), are plant compounds with antimicrobial, antioxidant, and anti-inflammatory properties (Álvarez-Martínez et al., 2021). Botanicals, phytogenics, and phytobiotics are terms often used interchangeably to denote a category of feed additives where the primary components are sourced from plants, including extracts, essential oils, and complex mixtures. However, the legal framework for feed additives

[1] Pathobionts are opportunistic microbes that emerge as a result of perturbations in the healthy microbiome due to complex interactions of various genetic, exposomal, microbial, and host factors that lead to their selection and expansion (Chandra et al., 2021).

[2] The use of antibiotics in animal feed has been prohibited over the years, for instance: Sweden (1986), Denmark, (1999), the European Union (2006), Korea (2011), the US (2017), and Canada (2018). The EU has also imposed limits on zinc in feed and routine antibiotic use since 2022.

in the EU, defined by Regulation (EC) 1831/2003, categorizes additives based on their functionality (e.g., technological, sensory, zootechnical additives) and includes specific dispositions about safety and claims.

While plant materials can be part of feed additive formulations, they can also be provided in other forms, such as supplements topping the feed, feed ingredients, or medicinal products, each following different legal frameworks (Franz et al., 2020). Given these nuances, the current thesis will favor the broader term "plant materials", allowing for a more flexible investigation into their potential benefits in animal nutrition and health.

Some plants materials can effectively manage PWD and ANE by either combating bacteria directly or indirectly through modulating gut microbiota or host immunity (Emami and Dalloul, 2021, Duarte et al., 2023b). Furthermore, the combination of modes of action may result in enhanced action against pathogenic bacteria resulting in more effective strategies (Bailey and Stokes, 2022, Canibe et al., 2022). Remarkably, most available data on the use of plant materials as in-feed non-antibiotic feed additives comes from combination strategies (Windisch et al., 2008, Rossi et al., 2020), often derived from commercial blends. Synergistic effects from bioactive constituents in plant extracts (or by-products of chemical reactions) are often cited as reasons for enhanced efficacy in numerous blends (Wagner and Ulrich-Merzenich, 2009). However, systematic research focusing on individual plants or substances and their interactions is still scarce (Rossi et al., 2020).

The aim of this chapter is to review the scientific literature on plant materials as antibacterial agents, with a particular focus on their combined use for enhanced efficacy in managing **PWD** in piglets and **ANE** in broiler chickens.

Definitions on Plant Based Products Used in Animal Nutrition and Health Literature

Phytochemicals: Naturally occurring compounds found in plants with potential biological activities. Examples: Flavonoids (quercetin, catechins), terpenes (limonene, pinene), organosulfurs (allicin, isothiocyanates), phenolic acids (caffeic acid, gallic acid), carotenoids (beta-carotene, lycopene). Notes: They are not regulated as feed additives themselves but fall under broader regulations for food/feed safety and health claims.

Botanicals: A broad term for entire or processed parts of a plants or manufacture products used in various applications, typically medicinal or cosmetics. Examples: herbal extracts and infusions, essential oils (e.g., oregano, thyme, peppermint, eucalyptus), spices (e.g., turmeric, ginger, peppers), plant-based powders (e.g., seaweed, alfalfa), blends. Notes: When used as feed additives, botanicals fall under specific regulations.

Phytogenics: Plant-derived feed additives used to promote animal health and performance. Examples: herbs (green tea, chamomile, ginko, dandelion and marigold), botanicals, essential oils and their components (e.g., oregano, thyme, cinnamon, thymol, carvacrol, cinnamaldehyde), and oleoresins (different from essential oils, volatile + non-volatile compounds, pigments, waxes, carotenoids, phenolics, etc. eg black pepper, cinnamon, ginger, capsicum oleoresins). Notes: Phytogenics is used in the literature but does not correspond to a category as zootechnical feed additives in the EU legislation.

Phytobiotics: Often used interchangeably with phytogenics, recently favored to emphasized claims on gut microbiota modulation and gut health promotion.

Plant materials: A general term encompassing various forms of plant-based products used in animal nutrition and health. Examples: All the above (phytochemicals, botanicals, phytogenics, phytobiotics), as well as other plant-based ingredients like grains, legumes, and oilseeds with functional properties. Notes: This is the most inclusive term and covers a wide range of products subject to various regulations depending on their specific category and intended use.

1.2 PIG POSTWEANING DIARRHEA AND BROILER NECROTIC ENTERITIS: ETIOLOGY, PATHOPHYSIOLOGY AND CONTROL

1.2.1 Pig Postweaning Diarrhea

Pig PWD affects piglets within the first two weeks after weaning and manifest as profuse diarrhea, dehydration, and weight loss (Fairbrother et al., 2005). It is a substantial cause of morbidity and mortality in young pigs, resulting in economic loss due to the cost of treatment, impaired growth, and animal losses (Rhouma et al., 2017b). Weaned piglets face challenges such as litter separation, environmental and dietary changes, and the establishment of new social hierarchies (Edwards et al., 2020). These stressors often result in reduced feed intake, depression, and lethargy, which may coincide with or contribute to the development of gut dysfunction and PWD (Gresse et al., 2017).

The characteristics of gut dysfunction during PWD in piglets have been extensively reviewed in the studies of Lallès et al. (2004) and Heo et al., (2013). This dysfunction is characterized by villus atrophy, depressed enzyme activity, impaired digestion, reduced absorption, local inflammation, mucosal disorders, oxidative stress, and increased permeability. Although PWD is recognized as a multifactorial phenomenon, the primary etiological agent is an ETEC infection (Francis, 2002). However, other infectious agents such as *Salmonella*, *Rotavirus*, and *Lawsonia intracellularis* may also contribute to its development (Eriksen et al., 2023).

Gut dysbiosis (Gresse et al., 2017) and nutrition (Gao et al., 2019a) have also been linked to development of PWD. During PWD, a significant reduction in bacterial diversity occurs, characterized by a decrease in *Lactobacillus* species and an increase in Proteobacteria (Gresse et al., 2017). This gut dysbiosis, along with the gut dysfunction, are believed to be central to the pathogenesis of the condition. Moreover, the protein type and content of the feed are thought to influence the development of PWD (Rist et al., 2013, Gao et al., 2019a). Due to insufficient digestion or absorption, excessive protein reaching the large intestine can promote the growth of pathogenic bacteria and the formation of potentially toxic fermentation byproducts (e.g. branched-chain fatty acids and biogenic amines) (Rist et al., 2013, Gao et al., 2019a).

Furthermore, the age of the pig at weaning has also been highlighted as a factor influencing the development of PWD (Sørensen et al., 2009). The gastrointestinal digestive capacity is connected to the age of the pig (Pluske et al., 2018). The stomach plays a crucial role as the first line of defense against potential pathogenic infections. Thus, high pH and reduced stomach emptying in piglets after weaning have also been linked to pathogen proliferation and PWD (Heo et al., 2013).

The interplay between the pig, its diet, and gut microbiota is crucial for understanding PWD. However, our current knowledge is evolving, particularly in refining causal relationships and mechanistic insights of disease onset (Bailey and Stokes, 2022).

Enterotoxigenic Escherichia coli

E. coli are facultative anaerobes and usual inhabitants of the pig gut microbiome (O'Neill et al., 2023). However, some strains of *E. coli* have evolutionarily acquired a set of virulence factors (e.g., adhesins, enterotoxins), which expression is associated with pathogenicity

(Escobar-Páramo et al., 2004). While there are various enteric *E. coli* pathotypes that affect both humans and animals (Pakbin et al., 2021), the enterotoxigenic is the one primarily associated with PWD in pigs. During PWD, infection occurs by ingestion of ETEC that colonizes and proliferates in the small intestine followed by bacterial attachment to receptors on enterocytes by specific adhesins (Francis, 2002).

In pigs, ETEC isolates produce any of five different fimbriae: K88 (**F4**), K99 (**F5**), 987P (**F6**), F41 (**F7**) and F18 (Francis, 2002). The fimbrial types F18 and F4 are commonly associated with PWD (Nguyen et al., 2017). Genetic markers, such as single nucleotide polymorphisms on genes like MUC4, MUC13, and FUT1, have been linked to ETEC resistance or susceptibility, though individual response variability remains (Luise et al., 2019b). Among the toxins produced by ETEC strains are heat labile enterotoxin (**LT**), heat stable enterotoxin type A (**STa**), heat stable enterotoxin type B (**STb**), Shiga toxin type 2e (**Stx2e**), and enteroaggregative *E. coli* heat-stable enterotoxin 1 (**EAST1**) (Francis, 2002).

The pathogenesis is complex and still being elucidated (Joffré et al., 2023). Nevertheless, some aspects of the disease pathogenesis have been demonstrated to play a role for disease onset (**Figure 1.1**)**.** Firstly, ETEC is ingested and needs to survive gastric digestion to reach the small intestine (Fairbrother et al., 2005). Under suitable conditions, ETEC colonizes and proliferates in the small intestinal mucosa. The degree of ETEC colonization determines whether the infection results in disease (Fairbrother et al., 2005). Diarrhea is ultimately induced by enterotoxins produced by the colonizing ETEC (Luppi et al., 2016). Specifically, the release of heat-stable and heat-labile enterotoxins activate cyclic nucleotide production (STs increase cyclic guanosine monophosphate; cGMP, while LT increase cyclic adenosine monophosphate; cAMP), which alter enterocytes function causing a net efflux of water and electrolytes into the intestinal lumen (Fairbrother et al., 2005, Pakbin et al., 2021). Furthermore, enterotoxins can downregulate the expression of thigh junction proteins and alter immune responses (Pakbin et al., 2021). Recent research shows that ETEC can sense various environmental stimuli in the host and modulate the expression of its virulence genes to cause infection (Joffré et al., 2023).

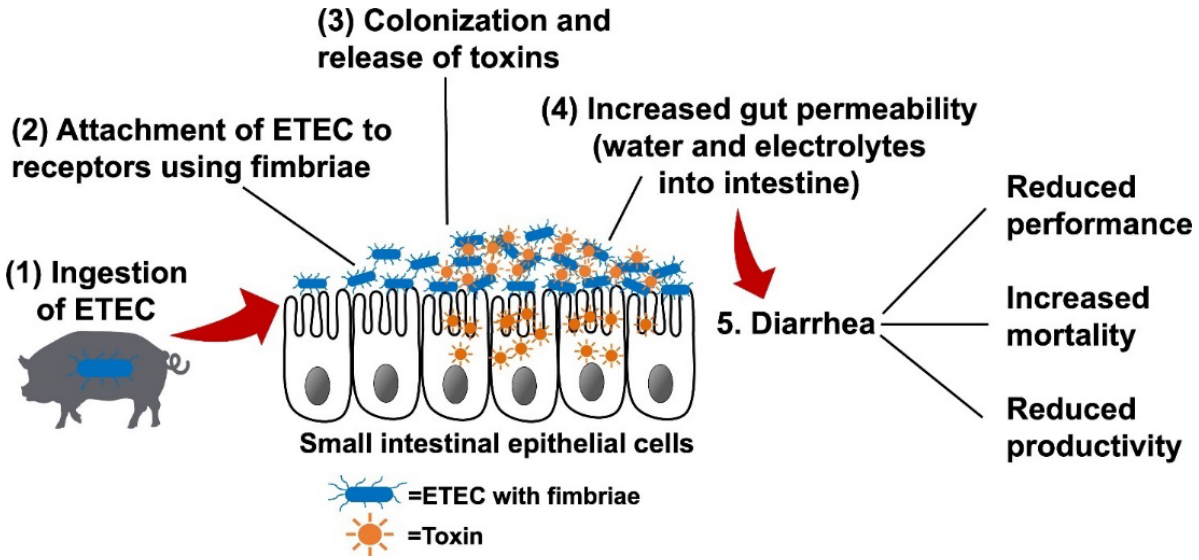


Figure 1.1 Summary of the pathogenesis of postweaning diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC).(1) Pigs ingest ETEC, which survives gastric digestion (2) Once in the small intestine, ETEC expresses fimbrial adhesins to adhere to epithelial cell receptors. (3) After establishment, ETEC produces enterotoxins. (4) Enterotoxins cause water and electrolyte loss into the intestinal lumen, increasing gut permeability. (5) Diarrhea ensues leading to poor performance, reduced productivity, and increased mortality. Extracted from: Kim et al. (2022) (CC BY 4.0).

The prevalence of AMR-associated resistance genes in ETEC has been a longstanding and evolving issue worldwide, with a notable increase in multidrug-resistant (**MDR**) strains over the past decades (Barros et al., 2023). Continuous use of antimicrobials may lead to the development of pan-resistance, including resistance to vital drugs like fluoroquinolones and cephalosporins (Barros et al., 2023). High rates of AMR against aminoglycosides, sulfamethoxazole, tetracycline, trimethoprim, and ampicillin have been detected in Danish isolates (García et al., 2020). It has been suggested that plasmids encoding virulent enterotoxins and those associated with antibiotic resistance are commonly transferred together (Barros et al., 2023).

Traditional Control Measures: Postweaning Diarrhea

Control and prevention strategies for PWD focus on achieving a direct antibacterial effect against ETEC, improved animal robustness, and neutralizing enterotoxins. Antibiotics[3] and pharmacological levels of zinc oxide have been used to control PWD and improve performance in conventionally raised piglets. Cromwell (2013) provided an overview of the effects of in-feed antibiotics with data collected in the US between 1960 and 1982. Pigs receiving antibiotics had an improvement in weight gain of 28% in clean facilities and up to 55% under unclean settings (Cromwell, 2013). Furthermore, the mortality rates could be reduced from ~16% to 3% when incorporating in-feed antibiotics (Cromwell, 2013).

The degree of impact of in-feed antibiotics varies according to the challenges presented to the animals, but the improvements in growth performance and reduced mortality are widely acknowledged. Yet, the exact mechanism of action is still not completely understood. It has been suggested that in-feed antibiotics inhibit the growth of the gut microbiota, thereby reducing competition for nutrients utilization with the host and reducing the maintenance costs of the gastrointestinal system (Gaskins et al., 2002). Furthermore, inhibition of subclinical infections, reduction of detrimental microbial metabolites concentration (e.g., ammonia or bile conjugates), and enhanced uptake of nutrients due to a histologically thinner gut have been observed in antibiotics-treated animals (Brüssow, 2015).

Supra-nutritional[4] supplementation of ZnO ranging from 1000 to 3000 mg/kg in weaner diets have resulted in reduced diarrhea and increased postweaning growth and intake (Sales, 2013). These links were discovered in the late 1980 and since then, it became another generalized practice in the industry and an effective alternative to in-feed antimicrobials[5] (Patience and Ramirez, 2022). The mechanisms are also still elusive, but it has been suggested that similar to subtherapeutic doses of antibiotics, a reduction in selected bacterial populations (*Lactobacillus* in particular) accompanied by the consequential reduction in fermentation of nutrients provide the piglet with more available nutrients for absorption (Højberg et al., 2005). Immunomodulation and improvement in oxidative status has been suggested as well as a mode of action of ZnO (Bonetti et al., 2021). The timing is another factor for the effectiveness in both strategies as the evidence indicates that extended use of both antibiotics or ZnO are either ineffective or even detrimental (Patience and Ramirez, 2022).

[3] An important distinction should be made between antibiotic treatment and in-feed antibiotic usage. In-feed antibiotic usage, a practice still prevalent in several parts of the world, involves incorporating subtherapeutic dosages of antibiotics into animal feed. The praxis has been banned in the EU since 2006.
[4] Also known as pharmacological or medical doses of ZnO.
[5] In the EU, the use of zinc oxide increased significantly after antibiotic use was restricted.

The associations between in-feed antibiotics and ZnO with antimicrobial resistance and environmental pollution have been widely recognized[6] (Holmes et al., 2016, EMA and EFSA, 2017). The use of antibiotics in livestock systems has diminished, in part thanks to prohibitions. So far, the use of supra-nutritional ZnO has been banned in the European Union since 2022 (Patience and Ramirez, 2022). Data[7] from 2021 to 2022 revealed a 7.6% increase in antibiotic use in weaners (**Figure 1.2**), largely attributable to increased neomycin consumption. Notably, this rise occurred concurrently with the EU-wide ban on medical zinc oxide in pigs, implemented in June 2022 (Duarte et al., 2023a).

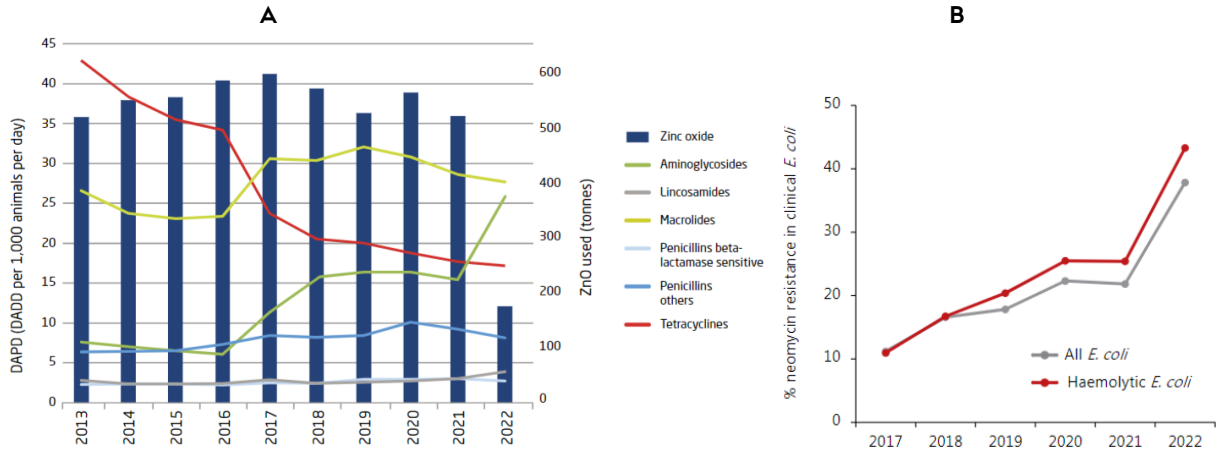


Figure 1.2 Increase in neomycin usage and resistance in weaners after the EU zinc oxide ban.(A) Antimicrobial usage by weaned pigs in Denmark (2013-2022). (B) Prevalence of neomycin-resistance among porcine clinical *E. coli* isolates in Denmark (2017-2022). Adapted from: Duarte et al. (2023a).

1.2.2 Avian Necrotic Enteritis in Broiler Chickens

In broiler chickens, ANE commonly occurs around 2 to 6 weeks of age in either clinical (acute) or subclinical (chronic) forms (Emami and Dalloul, 2021). Ruffled feathers, depression, diarrhea, huddling, anorexia, sternal recumbency, and sudden flock mortality are symptoms of severe clinical forms, accompanied by necrosis of the mucosal lining (To et al., 2017). Poor growth performance due to impaired digestive function characterizes the subclinical form of disease, causing the greatest economic impact in poultry production (Van Immerseel et al., 2009, Abd El-Hack et al., 2022). Also recognized as a multifactorial disease, the main etiological agent behind ANE is an infection by a pathogenic *Clostridium perfringens*. Nonetheless, predisposing factors (e.g., *Eimeria* infection, antimicrobial withdrawal, stress, and diet among others) must be present to allow the pathogenic *C. perfringens* to induce disease (Yang et al., 2019a).

Clostridium perfringens Type G (netB+)

C. perfringens is a Gram-positive anaerobic spore-forming bacterium, common inhabitant of poultry systems and the chicken's gut (Immerseel et al., 2004). The *C. perfringens* isolates are

[6] In the case of ZnO the connection with antimicrobial resistance is still under scrutiny, reasons for banning in the EU were mostly related to accumulation of Zn yielding environmental toxicity.
[7] In 1995, Denmark established DANMAP, the first systematic and continuous program to monitor antimicrobial drug usage and resistance.

classified following recently updated toxinotypes. Thus, *C. perfringens* typing A to G is assigned depending on their ability to produce one or combinations of the toxins: α-toxin, β-toxin, ε-toxin, ι-toxin, *C. perfringens* enterotoxin (cpe) or necrotic enteritis beta-like (NetB) toxin. The type G strains, producing α-toxin and NetB toxin but not β-toxin, ε-toxin, or ι-toxin, are primarily responsible for causing host-specific ANE in chickens (Rood et al., 2018).

Diseased birds display increased intestinal counts of *C. perfringens* (10^6-10^9 cfu/g), yet, pathogenesis crucially involves the NetB toxin (Dahiya et al., 2006, Timbermont et al., 2010). Barbara et al. (2008) reported the displacement of commensal *C. perfringens* by *netB*+ isolates during disease onset thanks to the secretion of bacteriocins. Further, research has shown that horizontal transfer of NetB toxin-encoding plasmids into commensal strains can also induce disease (Lacey et al., 2017). Indeed, NetB toxin has been proven essential for the experimental reproduction of the disease (Pedersen et al., 2008). Field data, however, presents a complex picture, i.e., *netB*+ isolates are found in both affected and healthy chickens (albeit less commonly), *netB*+ *C. perfringens* is not always present in confirmed cases, and *netB*- isolates also occur during the disease (Lee and Lillehoj, 2021).

It has been suggested that the ability of pathogenic *C. perfringens* to thrive in the chicken's intestine is closely linked to the intestinal events and nutritional dynamics to which the chickens are exposed (**Figure 1.3**) (Moran, 2014, M'Sadeq et al., 2015) . *Eimeria* infections are linked to ANE, the intestinal damage caused by coccidia is a key predisposing factor (M'Sadeq et al., 2015, Gharib-Naseri et al., 2019), as it enables the overgrowth of *C. perfringens* type G. During an *Eimeria* infection, intestinal damage causes proteins to leak into the intestinal lumen, providing a nutrient-rich substrate that fosters *C. perfringens* proliferation and toxin production (Van Immerseel et al., 2009). Excessive enteric mucogenic activity during coccidiosis is also favorable for *C. perfringens* overgrowth (Abd El-Hack et al., 2022).

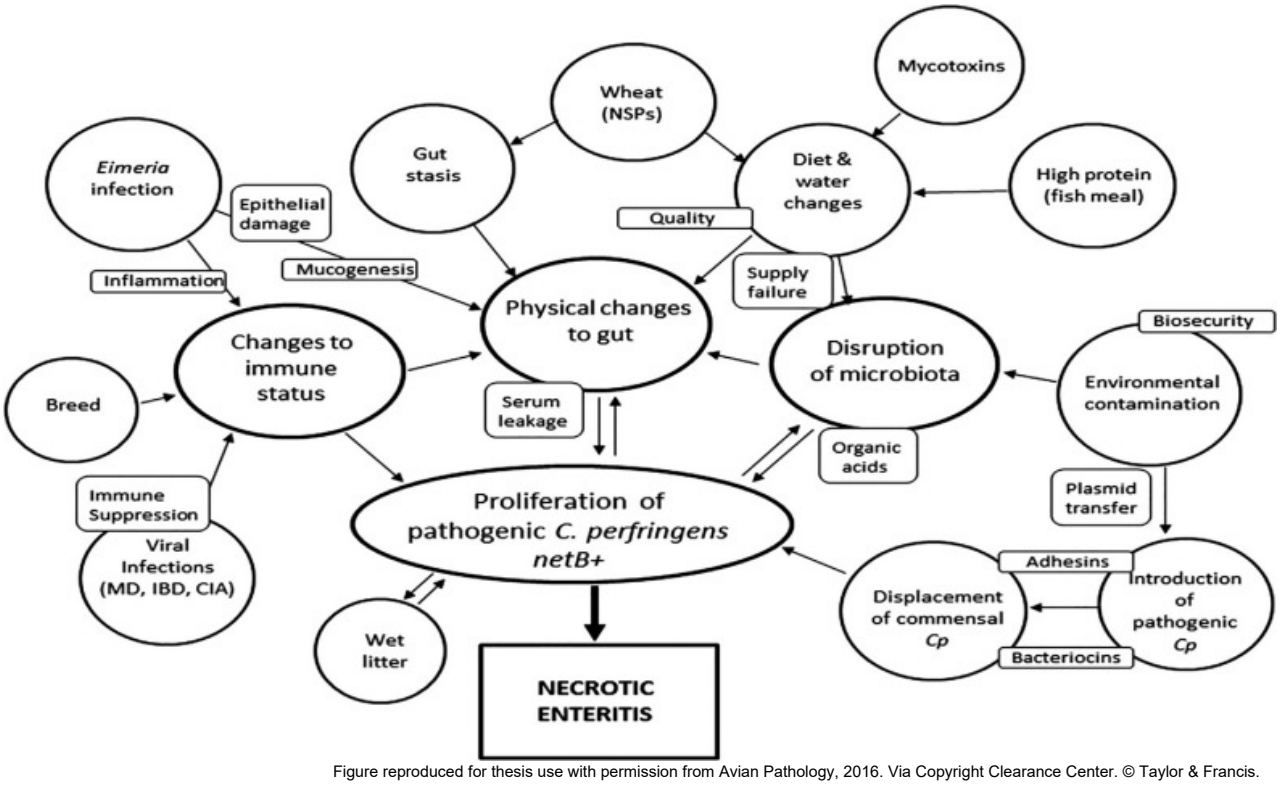


Figure 1.3 Summary of factors involved in the development of necrotic enteritis in chickens. Abbreviations: CIA (Chicken Infectious Anemia), Cp (Clostridium perfringens), IBD (Infectious Bursal Disease), MD (Marek's Disease), NSPs (Non-Starch Polysaccharides). Extracted from Moore, 2016.

The use of rye, oats, barley, and wheat-based diets has been shown to be predisposing for development of ANE (Annett et al., 2002). These grains have prominent levels of soluble non-starch polysaccharides (**NSP**), which increase the viscosity of the digesta. A higher increase in viscosity increases transit time and mucin production, both factors contributing to *C. perfringens* proliferation (Shojadoost et al., 2012, Moore, 2016). Increased water consumption resulting from non-starch polysaccharide (NSP) intake, and the subsequent development of wet litter, has been shown to promote environmental *C. perfringens* sporulation, leading to disease outbreaks in the field (Moore, 2016).

Diets high in ingredients of animal-origin, particularly fish meal, predispose to ANE due to the *C. perfringens* ability to readily digest and utilize these proteins (Shimizu et al., 2002, Shojadoost et al., 2012). The abundance of glycine in fish meals stimulates *C. perfringens* proliferation and α-toxin production (Dahiya et al., 2005). In general, high protein diets provide a rich substrate and can increase the gastrointestinal pH, thus creating an environment favorable for *C. perfringens* to thrive and induce disease (Moore, 2016).

Other factors that alter either the immune status or the gut microbiota have been shown to influence ANE outbreaks. The presence of mycotoxins in the diet, which impair intestinal microbiota, has also been linked to ANE development (Antonissen et al., 2015). Viral infections such as Marek's disease, infectious bursal disease and chicken anemia compromise the immune status of chickens potentially also leading to ANE (Hoerr, 2010, Moore, 2016).

Traditional Control Measures: Broiler Necrotic Enteritis

Historically, the broiler industry has also relied on the use of in-feed antibiotics. The restrictions on its use also resulted in a spike on the reports of ANE (Wierup, 2001, Williams, 2005). Improvements in hygiene, management and nutrition have contributed to mitigating challenges, but another player here is the use of ionophore coccidiostats (Frederiksen et al., 2024). Given the connection of ANE with *Eimeria* infections, the use of coccidiostats has been an effective tool for controlling ANE.

Coccidiostats (ionophores, synthetics or combinations) are used for the control of *Eimeria* infections as a prophylactic measure (Chapman et al., 2010). Although efforts have been made to reduce their use in broiler feed, their utilization remains widespread (Mesa-Pineda et al., 2021). In general, in the absence of conventional antibiotic growth promoters, broilers perform well under good hygienic conditions when fed an ionophore (Williams, 2005). Although coccidiostats are highly effective against coccidiosis, their efficacy against ANE is variable, being reduced especially under poor hygiene, husbandry and nutrition (Chapman et al., 2010).

Coccidiostats provide protection against ANE through a similar mode of action to that proposed for conventional in-feed antibiotics, i.e., by reducing the population of gram-positive bacteria, allowing for increased nutrient availability for absorption. However, controversy exists regarding the use of ionophores coccidiostats, which are indeed a class of antibiotics, not necessarily relevant for human medicine, but that may also contribute to development of antimicrobial resistance (Frederiksen et al., 2024). Currently, coccidiostats are generally approved for use in broiler feed, notwithstanding some industries participate on voluntary antibiotic-free schemes (Mesa-Pineda et al., 2021).

1.2.3 Postweaning Diarrhea and Necrotic Enteritis in Organic Systems

Organic farming systems face challenges with PWD and ANE, which are exacerbated by restrictions inherent to organic regulations. For example, PWD remains the primary gastrointestinal disorder affecting pigs in these systems, despite older weaning ages typically employed in organic farming (Leeb et al., 2014). Additionally, the use of ionophore coccidiostats is restricted in organic broiler systems, complicating the management of coccidiosis and ANE (Souillard et al., 2019).

A significant limitation in organic systems lies in the quality of the diets, which face various restrictions related to the use of ingredients and feed additives (Lindgren et al., 2014). Minimally processed plant materials would therefore be preferred in organic systems for provision of functional effects.

Conventional systems achieve optimal protein levels using high-quality, often preprocessed protein sources that are unavailable or prohibited in organic production (Blavi et al., 2021). Additionally, synthetic amino acids are often added to ensure the provision of essential amino acids without increasing the overall crude protein content— a practice not permitted under organic standards (Blair, 2018). Consequently, in organic farming, meeting the necessary levels of essential amino acids inevitably results in an excess of crude protein, which can contribute to the development of PWD or ANE.

1.2.4 Nutritional Alternatives for Prevention of Enteric Diseases

Due to the shared features in disease pathogenesis of PWD and ANE, and their association with the use of conventional antimicrobials, similar strategies have been used to find alternatives to PWD and ANE. Alternative strategies include management procedures, breeding programs and nutritional/dietary strategies (Patience and Ramirez, 2022). The use of nutritional or dietary strategies to control PWD and ANE has been extensively investigated (Rist et al., 2013, M'Sadeq et al., 2015, Diaz Carrasco et al., 2016, Morgan, 2017, Rhouma et al., 2017a, Gresse et al., 2017, Gao et al., 2019a, Girard and Bee, 2020, Jackman et al., 2020, Gomez-Osorio et al., 2021, Abd El-Hack et al., 2022).

Preventive strategies for PWD or ANE can be developed to exert a direct antimicrobial action against ETEC or *C. perfringens* (type G) or to diminish the infection by modulating the gut microbiota or host immunity. Several of these strategies have long been studied, for example the use of several probiotics has been assessed in multiple studies in both PWD (Barba-Vidal et al., 2018, Wang and Gänzle, 2019, Hansen et al., 2022) and ANE (Abd El-Hack et al., 2020, Yaqoob et al., 2022). Several studies report positive outcomes, but the responses are often inconsistent (Patience and Ramirez, 2022) and largely dependent on the initial sanitary conditions. A summary of the most frequently investigated strategies is presented in **Figure 1.4**.

Because of the inconsistency and inefficacy of several strategies, authors argue that combining strategies may be a better approach for replacing antibiotics in monogastrics (Fan and Archbold, 2015). However, the possibility of interactions, including synergies and antagonism, warrants detailed knowledge of their modes of action.

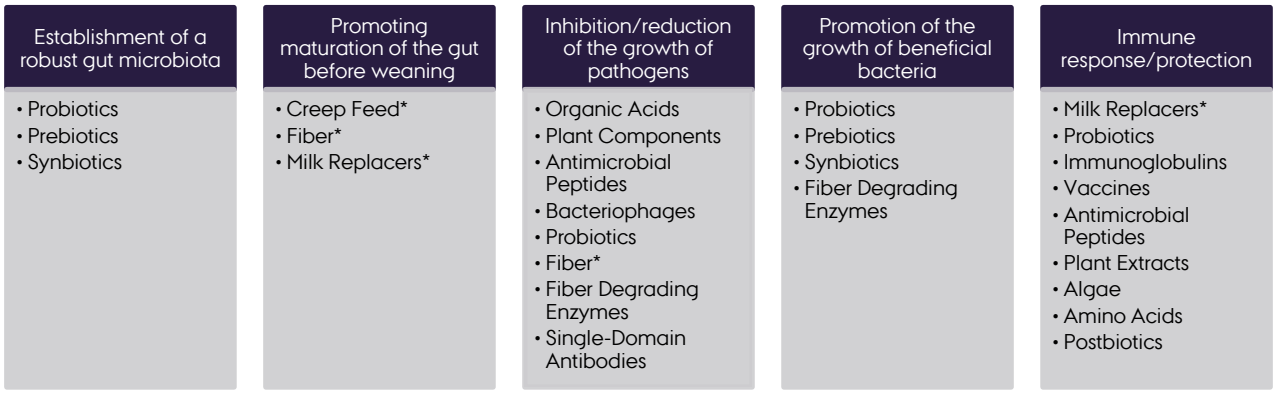


Figure 1.4 Proposed modes of action of various strategies to prevent piglet post-weaning diarrhea and chicken necrotic enteritis. *Apply only to piglets. Based on Canibe et al. (2022) and Abd El-Hack et al. (2022).

1.3 ANTIBACTERIAL PLANTS AND THEIR COMBINATION TO TARGET GASTROINTESTINAL PATHOGENS

Plant based products functional, including botanicals, phytogenics, or phytobiotics encompass a diverse array of botanical derivatives provided to animals for additional nutritional benefits. According to Franz et al. (2020), phytogenic feed additives can be categorized based on the anatomical part of the plant used, such as herbs (leaves and flowers) and spices (seeds, fruits, bark, or roots known for their strong flavors or aromas). They can further be classified by their extraction method (Franz et al., 2020), including essential oils (obtained through distillation of volatile substances), oleoresins (extracted using non-aqueous solvents), and extracts/tinctures (produced through maceration, percolation, or supercritical CO^2 extraction). Furthermore, plant residues, byproducts, and waste materials from industrial processing made another category (Franz et al., 2020).

The effectiveness of plant materials on supporting animal health is attributed to their bioactive components. Plant materials may contain one or multiple bioactive substances derived from one or several plant species. Therefore, their effects might stem from a single substance or a combination of them, potentially offering a range of therapeutic benefits (Wagner and Ulrich-Merzenich, 2009). The bioactive substances in plants come from an array of compounds known as phytochemicals which provide the plant with protection against stress, predation, and infection (Forgie et al., 2019). Phytochemicals, categorized by Rossi et al. (2020) into phenolics (e.g., apigenin, quercetin, curcumin, resveratrol), organosulfurs (e.g., allicin), terpenes (e.g., eugenol, thymol, carvacrol, artemisinin, capsaicin), and aldehydes (e.g., cinnamaldehyde, vanillin), have received significant attention in animal nutrition and health. Furthermore, the properties of plant phytochemicals impart colors, smells, and flavors, as well as dictate bioactivities and bioavailability within the GIT (Kemperman et al., 2010, Forgie et al., 2019).

Several phytochemicals have antibacterial activity, making them an attractive alternative to conventional antimicrobials for controlling PWD and ANE. Although the antibacterial properties of several plant materials are well established in vitro, translating these effects to in vivo conditions remains a challenge (Wenk, 2001). This gap highlights the importance of the substantial body of research dedicated to investigating the antibacterial effects of plant

materials or phytochemicals in piglets and chickens (Windisch et al., 2008, Franz et al., 2010, Lillehoj et al., 2018, Huang et al., 2018, Abdelli et al., 2021).

The combination of different plants and phytochemicals introduces complexity, as interactions among individual components within the plants or mixtures can influence the overall effect (Wagner and Ulrich-Merzenich, 2009, Canibe et al., 2022). On the other hand, it also provides major benefits when successful. Combination strategies that result in synergistic interaction can lower the inclusion of individual components due to their combined efficacy, reduce the risk of developing antibacterial resistance, and restore lost antimicrobial activity (Cheesman et al., 2017).

1.3.1 Plant Natural Products Targeting Enterotoxigenic *Escherichia coli*

Garlic

Garlic (*Allium sativum*), originally from central Asia and now globally cultivated, has been revered for millennia across various cultures for its culinary and medicinal properties (Rivlin, 2001). Garlic preparations have been used for treating leprosy, asthma, typhus, dysentery, cholera, influenza, and other infectious diseases in humans (Zhang et al., 2020, Sunanta et al., 2023). Furthermore, it is used in domestic animals as antimicrobial, antiparasitic and growth promoter (Chen et al., 2021).

The medicinal uses of garlic preparations are associated with antioxidant, hepatoprotective, anti-inflammatory, cardioprotective, anticancer, antidiabetic and antimicrobial activities (Tudu et al., 2022). Several of these properties have been attributed to phytochemicals, most of which are organosulfur compounds (**Figure 1.5**), from that ~ 80% corresponds to carbohydrates (mostly fructans), ~5.7% protein, ~3.4 % free amino acids (mainly arginine), ~2.3% organosulfur compounds, and ~1.5 % fiber, fatty acids, phenols and trace minerals (Zhang et al., 2020). Sulfur containing compounds give garlic not only its distinct organoleptic qualities, but also biofunctional properties (Martins et al., 2016). Garlic bulbs are also a source of vitamins (e.g., B complex and C), antioxidants, flavonoids, and minerals (e.g., P, K, and Se) (Lanzotti et al., 2014).

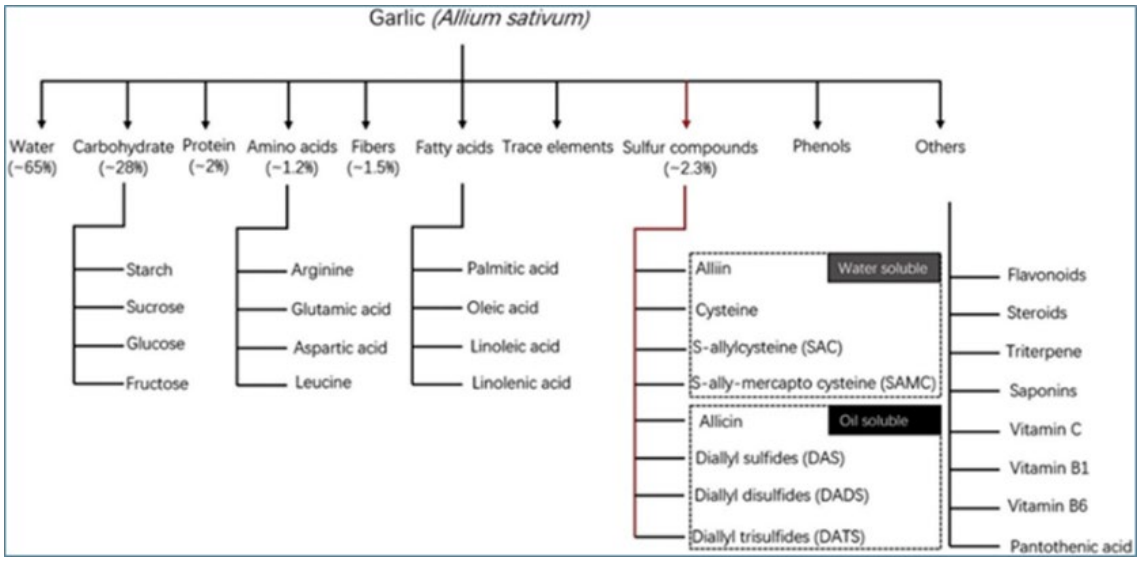


Figure 1.5 Major chemical components of garlic bulbs. Extracted from: (Zhang et al., 2020) (CC BY-NC-ND 4.0 DEED)

Garlic bulbs contain flavonoids, saponins, sapogenins, phenolic compounds, nitrogen oxides, amides (Martins et al., 2016). The bioactive molecules profile is largely dependent on preharvest (genotype, cultivation, cropping) and postharvest conditions (processing, storage, treatments), as reviewed by Martins et al. (2016) and Sunanta et al. (2023).

The main organosulfur compounds in intact garlic are γ-glutamyl-S-alk(en)yl-L-cysteines and S-alk(en)yl-L-cysteine sulfoxides (Figure **1.6**). In the latter group, S-allyl-L-cysteine sulfoxide (alliin), S-(trans-1-propenyl)- L-cysteine sulfoxide (isoalliin), and S-methyl-L- cysteine sulfoxide (methiin), and S-allyl cysteine (Bhatwalkar et al., 2021) are found. The most prominent organosulfur compound associated with garlic is allicin (diallyl thiosulfinate[8], $C_6H_{10}OS_2$), though no allicin can be found in garlic bulbs. Instead, allicin is produced from alliin by the enzyme alliinase upon crushing of plant cells (Reiter et al., 2020). In undamaged garlic tissues, alliin and alliinase are located in the cytoplasm and vacuole, respectively (Martins et al., 2016). When garlic cloves are crushed, the rapid transformation of alliin into allicin and other allyl thiosulfinates is triggered (Lawson and Gardner, 2005). In garlic powder preparations, exposure of the powder to water initiates this transformation, but only if alliinase is not damaged (Lawson and Hunsaker, 2018). Once formed, allicin undergoes conversion to secondary compounds, which is influenced by the temperature and pH (Salehi et al., 2019).

The bioactive molecules profile of garlic is largely dependent on preharvest (genotype, cultivation, cropping) and postharvest conditions (processing, storage, treatments), as review by Martins et al. (2016) and Sunanta et al. (2023). Genotype and growing conditions affect the organosulfur, phenolic content, flavonoids, proximal composition and alliinase content (Hirata et al., 2016, Wu et al., 2016, Petropoulos et al., 2018, Phan et al., 2019, Choi et al., 2020b). Sulfur application during cultivation particularly enhances organosulfur content and overall garlic quality (Martins et al., 2016). Therefore, sulfur fertilization increases alliin and other sulfur-containing metabolites like cysteine, cysteine sulfoxides, glutathione, and glucosinolates (Martins et al., 2016).

The chemical composition of garlic is subject to alteration by various post-harvest processing techniques, selected based on the intended use and desired properties of the final product. These techniques include, but are not limited to, drying, freeze-drying, blanching, roasting, frying, high-pressure processing, chemical treatment, and irradiation (Martins et al., 2016, Sunanta et al., 2023). Notably, processing parameters such as temperature, pH, and duration play a crucial role in determining the final chemical profile. For example, different drying processes have been reported to decrease the allicin (13% - 23%) and phenolic (45 - 57%) content compared to the fresh garlic (Sunanta et al., 2023). A known factor affecting the potential formation of allicin in garlic products is the denaturation of alliinase. Denaturation alliinase begins at 42 °C, achieving inactivation at 60 °C (direct exposure); however, a cycling drying regime from 40 to 60 °C has shown to preserved 91% allicin forming potential (Méndez Lagunas and Castaigne, 2008). Interestingly, storing temperature of 5 °C has shown to increase the amount of alliin, methiin and isoalliin up to 30%, which has been attributed to the transformation of γ-glutamyl peptides into sulfoxides (Ichikawa et al., 2006).

[8] PubChem CID: 65036, Allicin (162.3 g/mol) also known as allimin, allylthiosulfinate, allylthiosulphinic acid allyl ester, diallyl disulfide-oxide, thio-2-propene-1-sulfinic acid S-allyl ester.

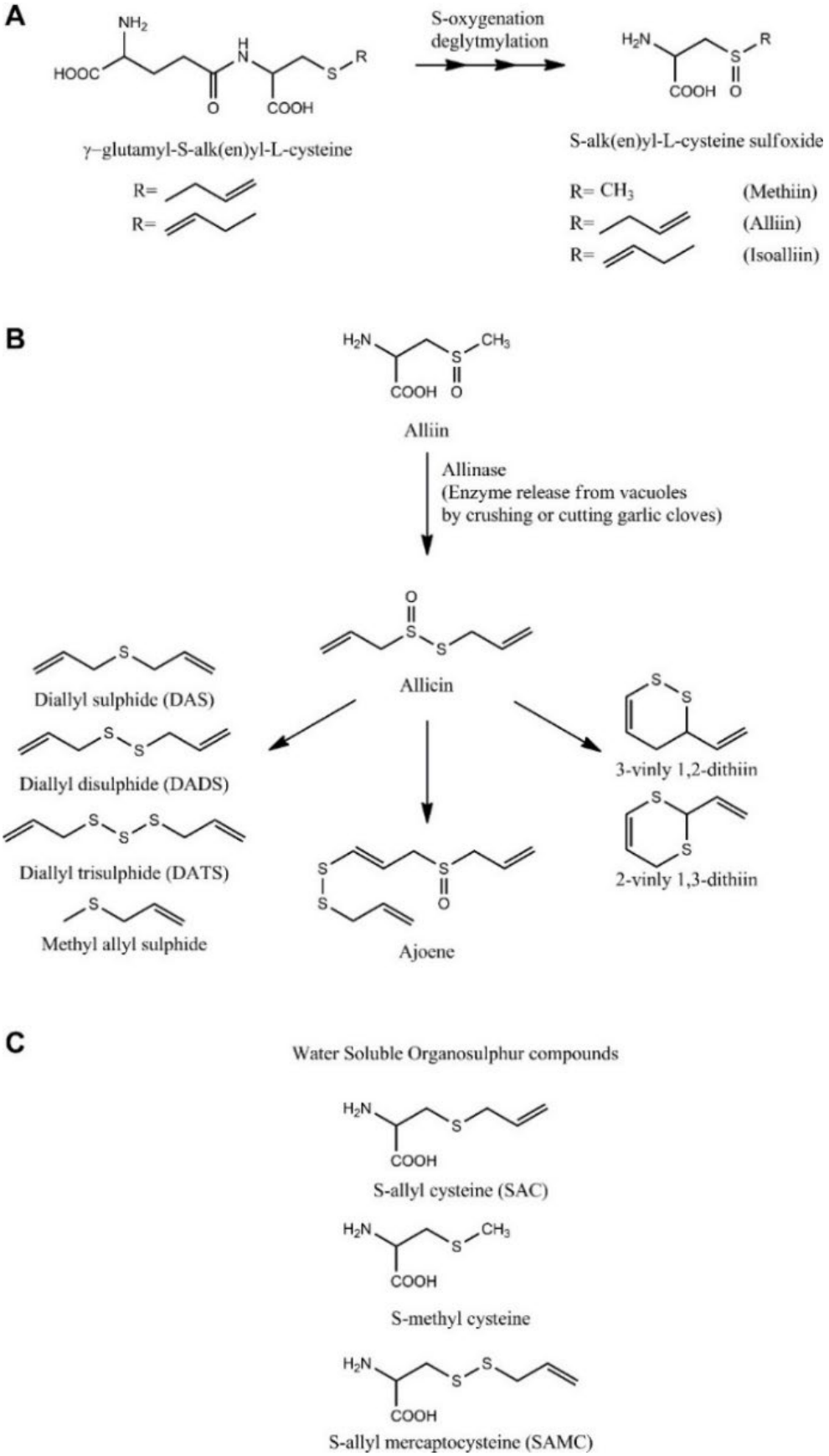


Figure 1.6 Organosulfur compounds of garlic: (A) Major organosulfur compounds in garlic cloves. (B) Upon tissue damage, in the presence of water, alliin is transformed into allicin by the action of alliinase. Allicin may undergo further degradation into several compounds. (C) Water soluble organosulfurs compounds, are also present in garlic or may form during aging or extraction. Extracted from: Bhatwalkar et al. (2021) (CC BY 4.0).

Extraction procedures result in concentration of active compounds with different techniques resulting in different chemical profiles (**Figure 1.7**). Freeze-drying fresh garlic cloves preserves its chemical composition; while low-temperature drying forms allicin and allyl sulfides (Staba et al., 2001). Steam distillation produces allyl sulfides; and maceration in oil creates products with vinyldithins, allyl sulfides, and ajoene (Staba et al., 2001). Hydroalcoholic short extraction yields tinctures with variable stability; and long maceration in ethanol converts allicin to various allyl sulfides, including S-allylcysteine (Staba et al., 2001).

Fresh garlic extract, garlic powder, and garlic paste undergo minimal processing, keeping most of garlic's natural compounds. Thus, the variability in the composition of these products is higher than in other processed products (Lawson and Hunsaker, 2018). Aqueous, ethanolic, and methanolic garlic extracts focus on extracting allicin, with ethanolic extraction yielding a higher allicin concentration (Bhatwalkar et al., 2021). Garlic oil has greater stability and contains mainly diallyl disulfide (**DADS**) and diallyl trisulfide (**DATS**) (Ross et al., 2001). Phytochemicals such as Z-10-devinylajoene, iso-E-10-devinylajoene, diallyl sulfide (**DAS**), DADS, DATS, and mixtures of diallyl sulfides can be concentrated and isolated through various extraction techniques (Bar et al., 2022).

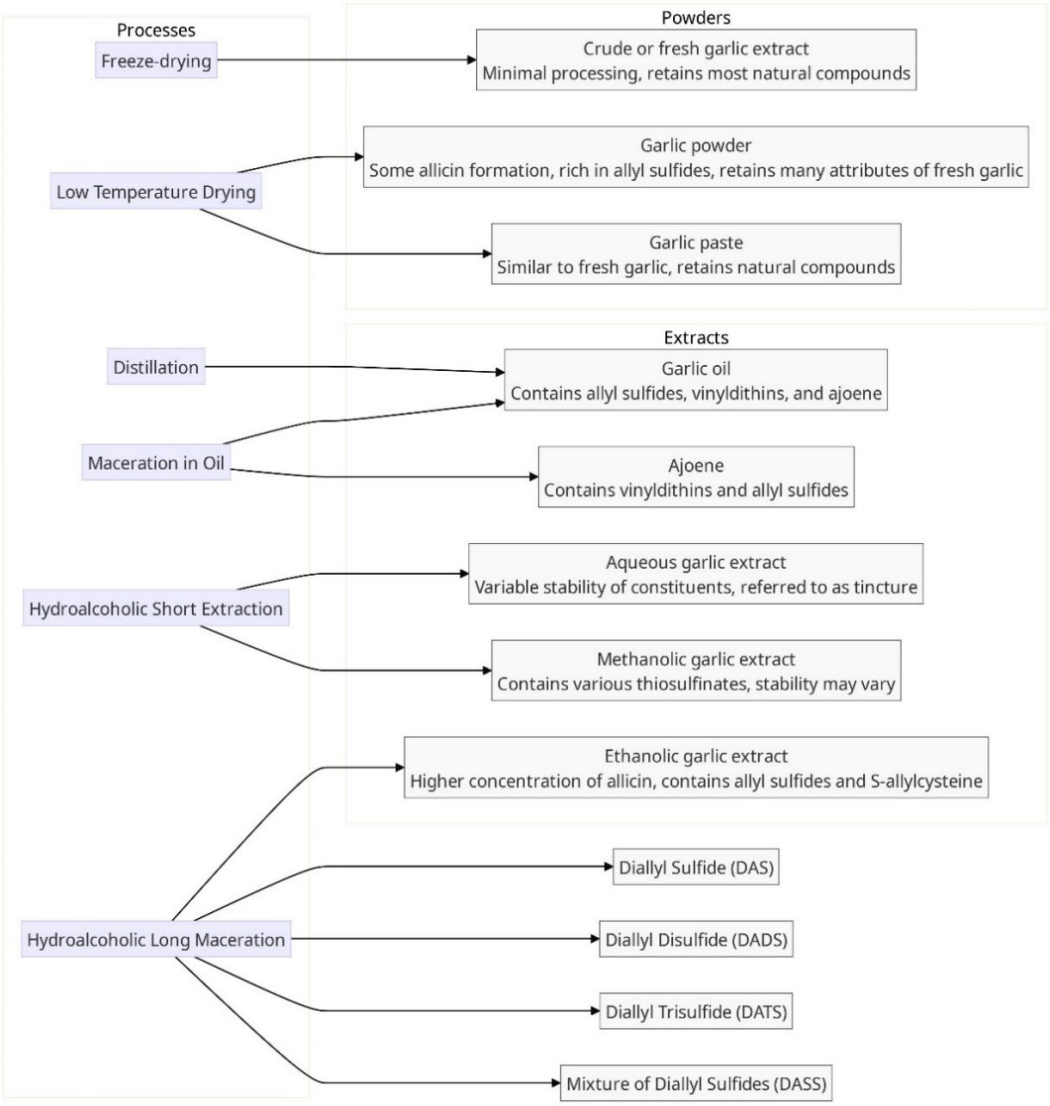


Figure 1.7 Summary of main methods for production of different garlic products. The different techniques used for extraction of garlic bulbs yield different products, resulting in varied physicochemical properties. Based on: Staba et al., 2001, Ross et al., 2001, Lawson and Hunsaker, 2018, Bhatwalkar et al., 2021.

Antibacterial Properties

The main antibacterial agent in garlic was first described in 1944, isolated as diallyl thiosulfinate ($C_6H_{10}OS_2$), and named allicin (Cavallito and Bailey, 1944a). Cavallito and Bailey described allicin as *more bacteriostatic than bactericidal* and *about equally effective against gram-positive and gram-negative bacteria*. Since then, several studies have reported the effectiveness of allicin against different bacteria, fungi, oomycetes, protozoa and virus (Reiter et al., 2017, Rouf et al., 2020). Interestingly, even though the allicin content is a good predictor of antimicrobial activity and its absence renders inactivity (Cañizares et al., 2004b, Borlinghaus et al., 2014), garlic extract is often a more effective antibacterial than pure allicin (Fujisawa et al., 2009). This suggests that other components in the garlic extract may synergize with allicin, enhancing its antibacterial efficacy. Cañizares et al. (2004a) observed for example a synergistic interaction between allicin and allyl-methyl thiosulfinate against *Helicobacter pylori*.

Allicin receives most attention, but other organosulfur compounds in garlic have also been found to possess antibacterial properties. These molecules include ajoene (Naganawa et al., 1996), S-allylcysteine, diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), and mixtures of diallyl sulfides (Bhatwalkar et al., 2021) (Figure 1.6). Yet, despite their antimicrobial properties, none of these compounds appear to be as effective an antibacterial agent as allicin (Borlinghaus et al., 2014). The relative effectiveness of allicin is comparable to conventional antibiotics (e.g., penicillin, ampicillin, annamycin, colistin), but allicin is considered to be effective against a broader spectrum of microorganisms than most antibiotics (Borlinghaus et al., 2014). Furthermore, allicin has a broad spectrum of cellular targets compared to antibiotics, thus making the development of resistance less likely (Müller et al., 2016, Ankri and Mirelman, 1999). However, the challenges associated with securing patents for natural antimicrobial compounds may have prevented the advancement of allicin into pharmaceutical products, as clinical efficacy trials require significant investments (Ankri and Mirelman, 1999).

Antibacterial Activity Against Escherichia coli

Garlic products and various garlic-derived organosulfurs have demonstrated significant antibacterial activity against different strains of *E. coli* (Bhatwalkar et al., 2021). Garlic powders, fresh garlic extract, and garlic paste are effective against commensal and pathogenic enteric bacteria, including the *E. coli* O157:H7 strain (Bhatwalkar et al., 2021). Additionally, fresh garlic extract exhibited antibacterial activity against MDR *E. coli* isolates from clinical and food samples both in vitro (Bhatwalkar et al., 2019) and in vivo assays (Farrag et al., 2019). Aqueous garlic extract has also shown inhibitory effects on ETEC in vitro (Arora and Kaur, 1999, El Astal, 2004). Similarly, garlic oil has been found effective against *E. coli*, as well as other pathogens in disk diffusion assays (Casella et al., 2013). However, Kim et al., 2004 found no inhibition of *garlic oil* against an *E. coli* B34 strain, which they attributed to the low solubility of the garlic oil in their assay. Furthermore, allicin itself has shown in vitro efficacy against *E. coli* in multiple instances (Müller et al., 2016, Marchese et al., 2016). The organosulfur compounds in garlic: DAS, DADS, and DATS, also exhibit in vitro antibacterial activity against *E. coli* O157:H7 (Yin and Cheng, 2003, Feng et al., 2014).

Antibacterial Activity of Garlic Components- Mode of Action

The main mechanism behind the antibacterial effect of most garlic products is suggested to be related to reactive sulfur species, primarily attributed to the characteristics of allicin (**Figure**

1.8). Allicin reacts readily with free thiols (–SH), targeting a wide range of cellular proteins and enzymes (Reiter et al., 2020). The thiol reactivity of allicin has been investigated since its discovery in the 1940s. Cavallito and colleagues described the reactivity of allicin to thiol groups and proved it by inactivation with cysteine (Cavallito and Bailey, 1944b, Borlinghaus et al., 2014). Thus, allicin disrupts cellular processes by binding to thiol groups on proteins.

The mode of action of allicin is known to be complex and multitarget. One molecule of allicin can potentially bind two thiol groups altering the structure and functionality of proteins (Borlinghaus et al., 2021). For example, allicin reacts with free thiols of glutathione (**GSH**), oxidizing GSH to S-allyl-mercapto-glutathione, which diminishes the cellular GSH pool and leads to intracellular oxidative stress (Müller et al., 2016). Furthermore, allicin interacts with crucial enzymes like succinic dehydrogenase, hexokinase, ribosomal proteins, DNA maintenance proteins and others, altering their functionality (Reiter et al., 2020, Borlinghaus et al., 2021). Thus, the action of allicin is mostly intracellular and most enzymes with available thiol groups are susceptible (Borlinghaus et al., 2014). Moreover, some enzymes not containing thiol groups have been also shown to be susceptible, but the mechanism of inactivation is unknown (Borlinghaus et al., 2014).

Nucleic acids and proteins synthesis processes have also been shown to be impaired by allicin exposure in *Salmonella* (Feldberg et al., 1988). Furthermore, allicin easily penetrates cell membranes and increases their porosity (Gruhlke et al., 2015, Miron et al., 2000). This facilitates the enhanced activity of conventional antibiotics when combined with allicin, resulting in a synergistic action (Gruhlke et al., 2015, Miron et al., 2000). Thus, allicin's effective inhibition of vital cellular enzymes, combined with its ability to enhance membrane permeability, underlines its significant impact on bacterial cell viability.

Allicin triggers complex cellular responses in bacteria to combat oxidative stress and damage. Key responses include the activation of heat shock proteins, antioxidant defenses, and metal stress responses, which help mitigate protein and DNA damage (Borlinghaus et al., 2014). Central to these defenses are redox buffer systems like GSH in Gram-negative bacteria and bacillithiol (BSH) in Gram-positive bacteria, crucial for maintaining redox homeostasis (Müller et al., 2016). Genes involved in DNA repair, protein folding, and antioxidant defense are upregulated, ensuring the repair of damaged cellular components (Reiter et al., 2020). Specific regulons, such as OxyR in *E. coli*, coordinate these responses regulating genes essential for oxidative stress resistance (Gruhlke et al., 2015).

The antibacterial mechanisms of other organosulfurs in garlic are less studied, but some mirror those of allicin, while others uniquely affect cell membranes. Organosulfides (e.g. DAS, DADS, DATS) also react with free thiol groups of proteins. However, lacking the sulfur bound oxygen make them potentially less reactive than allicin (Ross et al., 2001). Like allicin, the activity of garlic oil rich in organosulfides is diminished when treated with cysteine (Ross et al., 2001). Feng et al. (2014) reported an increasing antibacterial action of the diallyl sulfides according to the number of sulfur atoms (i.e., DAS < DADS < DATS). On the other hand, other compounds, like ajoene and DAS, display specific activities that disrupt cell membranes (Lu et al., 2011, Feng et al., 2014). These variations can be attributed to differences in molecular structure and reactivity, which influence how each compound interacts with bacterial cells.

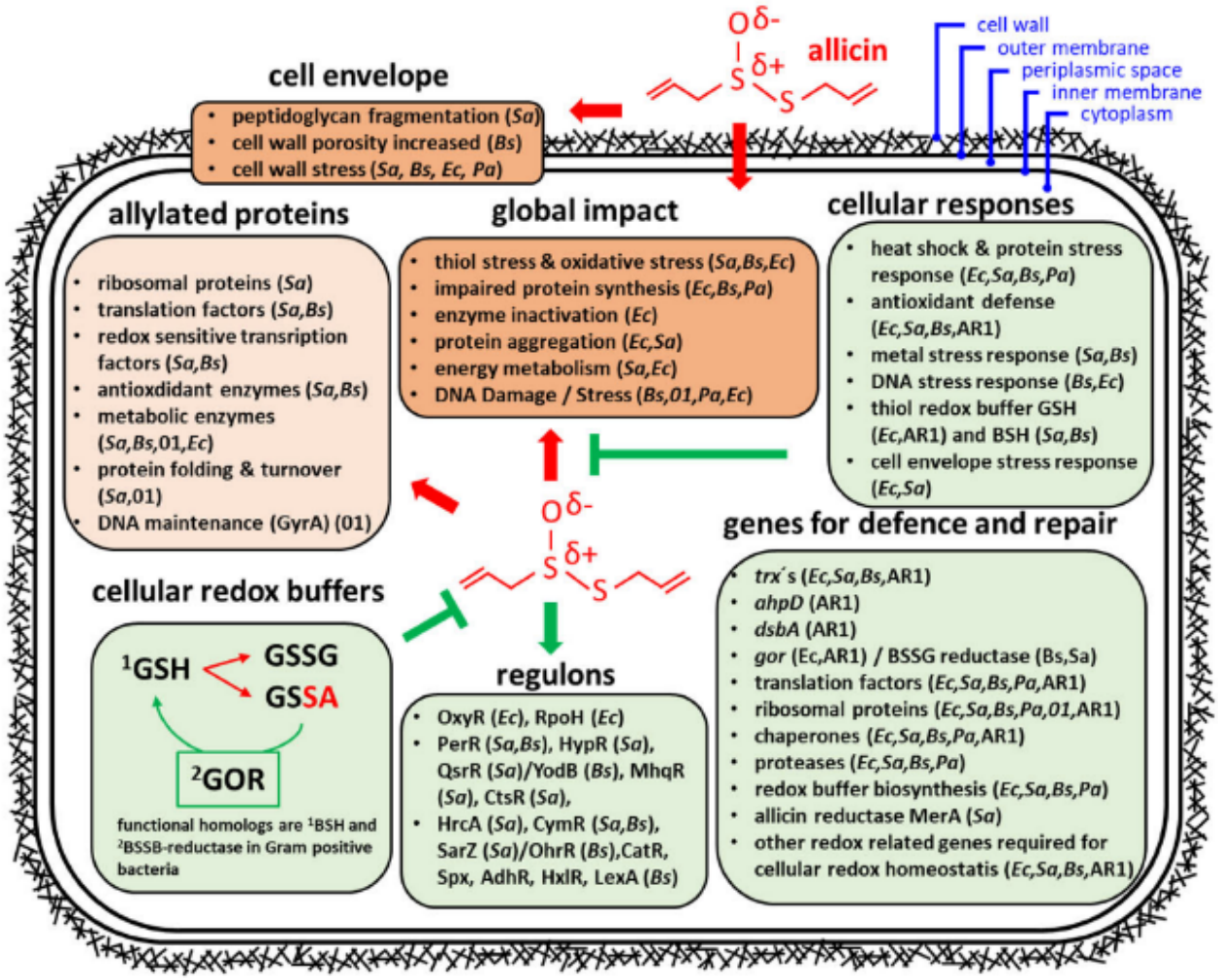


Figure 1.8 Summary of the effects of allicin on bacterial cells. The bacteria are: Ec = *E. coli* K12, Sa = S. aureus USA300, Bs = Bacillus subtilis strain 168, Pa = Pseudomonas aeruginosa PA01, 01 = P. fluorescens 01, AR1 = P. fluorescens AR1. Abbreviations are: Trx = thioredoxin; AhpD = alkylhydroperoxidase D; DsbA = disulfide bond protein A; GSH = glutathione; GSSG = glutathione disulfide; GSSA = S-allylmercaptoglutathione; BSH = bacillithiol; BSSB = bacillithiol disulfide; GOR = glutathione disulfide reductase; GyrA = DNA gyrase subunit A. The heat and oxidative stress regulons induced under allicin stress in different bacteria are indicated, that are either regulated by S-thioallylation of the redox-sensitive regulators (YodB, OhrR, HypR, CatR) or by other mechanisms, such as increased oxidatively damaged proteins (e.g., RpoH, HrcA, CtsR). Extracted from: Borlinghaus et al. (2021) (CC BY 4.0 DEED)

Garlic Products Against Pig Postweaning Diarrhea

Garlic products show promising effects on pig health and performance, particularly improving growth metrics (Ayrle et al., 2016, Chen et al., 2021) (**Table 1.1**). However, studies directly investigating their impact under experimental *E. coli* infection conditions remain limited. Garlic botanical (steamed distilled oil, 40% propyl thiosulfonates; 10 ppm feed) significantly reduced diarrhea scores, white blood cell counts, and inflammation markers in pigs experimentally infected ETEC F-18, while increasing ileal villi height (Liu et al., 2013). Fermented garlic powder improved ADG and immune responses in pigs challenged with *E. coli* lipopolysaccharide, with a notable reduction in fecal *E. coli* counts (Wang et al., 2011).

In non-challenge studies, fermented garlic powder (Yan and Kim, 2013), purple garlic powder (Rivera-Gomis et al., 2020), and garlic powder (Thuy and Ha, 2023) were found to improve average daily gain, feed conversion ratio, and nutrient digestibility, while significantly

reducing diarrhea incidence and fecal *E. coli* concentrations in pigs. However, also in non-challenge conditions, garlic powder (grain size 0.3–0.8 mm; 0.5% allicin; 0.3 g/kg BW/day) improved growth performance and clinical health but did not significantly reduce the PWD symptoms, nor fecal coliforms count (Ayrle et al., 2019). In a study using a water-feed deprivation challenge, garlic-derived DADS and DATS did not improve growth but increased villus height and ileal mucosa superoxide dismutase activity (Horn et al., 2017).

Recently, garlic oil (10 ppm feed) in combination with capsicum oleoresin was shown to reduce diarrhea severity while improving gut morphology and modulating immune response in weaned piglets experimentally infected with ETEC F18 (Wong et al., 2022). Furthermore, they observed that a lower dose of the same combination was not effective. Although the study did not assess the degree of interaction between capsicum oleoresin and garlic oil, the combination proved beneficial. Overall, while garlic products show beneficial effects, further research is needed to fully understand their efficacy for preventing PWD under varying conditions and challenges.

Table 1.1 Summary of recent studies using garlic products in piglets. : Controlled ETEC challenge studies, LPS: *E. coli* lipopolysaccharide. ↗ / ↘ : increased/decreased compared to untreated control.

Garlic product, inclusion	Main Findings	Reference
Garlic botanical 0.01 g/kg feed,	Diarrhea score ↘, frequency ↘, TNF-α ↘, haptoglobin ↘, white blood cell counts ↘, neutrophils ↘, ileal villi height ↗	(Liu et al., 2013)
Fermented garlic powder 2 g/kg feed, LPS	ADG ↗, fecal *E. coli* count ↘, rectal temperature ↘ after LPS challenge, improved immune response	(Wang et al., 2011)
Fermented garlic powder 0.5, 1 and 2 g/kg feed	0.5 g/kg: ADG ↗, ADFI ↗, nutrient digestibility ↗, 1-2g/kg: lymphocyte and RBC concentrations ↗, fecal *E. coli* concentration ↘	(Yan and Kim, 2013)
Garlic powder 2 g/kg feed	ADG ↗, FCR ↘, diarrhea incidence ↘ 7.43%, fecal *E. coli* counts ↘	(Nguyen et al., 2017)
Diallyl disulfide and Diallyl trisulfide 3.6 mg /kg BW (Oral gavage 6 days)	No differences in growth or diarrhea, villus height ↗, ileal mucosa superoxide dismutase activity ↗	(Horn et al., 2017)
Garlic powder 0.3 g/kg BW/day (top on feed daily)	ADG ↗, clinical score ↗, did not reduce PWD incidence and severity	(Ayrle et al., 2019)
Purple garlic powder 4 and 20 g/kg	4 g/kg: ADG ↗, no change in C-reactive protein and cortisol, cupric reducing antioxidant capacity ↗	(Rivera-Gomis et al., 2020)
Garlic oil 12% + capsicum oleoresin 0.3% 0.05, 0.1 g/kg feed	0.1 g/kg: diarrhea frequency↘, serum TNF-α↘, jejunal villi height ↗, gene expression of IL1B, PTGS2, TNFA in ileal mucosa ↘	(Wong et al., 2022)

Apple Pomace

Apples (*Malus domestica*) are the primary fruit produced in the EU, with an annual output of around 13 million tons, highlighting its significant agricultural productivity (Eurostat, 2024). Despite a global decrease in harvested areas, the production of apples in the EU has continued to increase, indicating improved agricultural efficiency (FAOSTAT, 2024). About 25-30% of the weight processed from these apples becomes residue, which includes peels, cores, seeds, calyxes, stems, and soft tissues (Lyu et al., 2020). This byproduct, known as apple pomace, is valued for its high content of polyphenols, dietary fiber, and organic acids (Nayak et al., 2020, Ezzat et al., 2022). The substantial production of apples in the EU leads to significant generation of apple pomace, offering both challenges and opportunities for utilization.

Apple pomace holds significant potential due to its content of valuable phytochemicals, yet its management presents considerable challenges (Bhushan et al., 2008). One major challenge is its high moisture content, ranging from 70 to 75%, which makes the pomace bulky and highly susceptible to rapid decomposition (Bhushan et al., 2008). This requires that any further uses or proper disposal consider the logistics of transportation and processing. Despite these challenges, the potential of apple pomace has been extensively explored for beneficial uses across the animal feed, pharmaceutical, food, and nutraceutical industries (Rana et al., 2015, Guil-Guerrero et al., 2016, Lyu et al., 2020, Ezzat et al., 2022).

Apple pomace contains significant nutritional and industrial potential due to its composition (**Table 1.2**). Fresh apple pomace has moisture content of 70-85%, when dried for storage or further use the moisture is reduced to 4-10% (Rana et al., 2015, Waldbauer et al., 2017). Most of the dry mass of apple pomace consists of skin-flesh (~95%) and seeds (~2-4%), these fractions are rich in cell wall polysaccharides and phenolic compounds (Rana et al., 2015). In the dry apple pomace, the main component is usually dietary fiber ranging from 27-82%, but generally around 40% (Waldbauer et al., 2017, Antonic et al., 2020a).

Based on the dietary fiber composition, approximately two-thirds correspond to insoluble fractions (cellulose, hemicellulose, lignin), while pectin accounts for most of the soluble fiber fractions (Skinner et al., 2018). Sugars (glucose, fructose, sucrose) content in dry apple pomace can vary widely, accounting for approximately 20-50% depending on the composition of the apples used, with fructose usually being relatively higher than other sugars present (Waldbauer et al., 2017, Antonic et al., 2020a). Lipids and protein content is usually below 5%, mostly contained in the seeds (Skinner et al., 2018). In the mineral composition, potassium is the most abundant at ~ 398.4–880.2 mg /100 g, alongside varying levels of calcium, phosphorus, magnesium, iron, zinc, copper, and manganese (Skinner et al., 2018, Antonic et al., 2020a).

Apple pomace is also regarded as an important source of phytochemicals and organic acids. After processing, more than 80% of polyphenols from the apple end in the pomace (Antonic et al., 2020a). The phenolic compounds in apple pomace come primarily from the seed and peel, principally chlorogenic acid and phloridzin (Rabetafika et al., 2014). The total amount of polyphenolics ranges from 0.17- 0.99% in dry apple pomace. Catechin, epicatechin, caffeic acid, procyanidin b2, hyperin and quercitrin have been also found in different quantities (Antonic et al., 2020a). Malic and citric acids are also organic acids found in apple pomace (Martău et al., 2021). Unripe apples generally have a higher content of starch and organic acids which can later degrade during maturation (Waldbauer et al., 2017). Ursolic acid, major component of the epicuticular wax in apples, is also present in apple pomace and has received

attention for its antioxidant, anti-inflammatory, anticancer, and antihepatotoxic activities (Antonic et al., 2020a).

Table 1.2 General composition of dry apple pomace.

Content	Value, %	Reference
Moisture Content (Fresh)	70-85	Rana et al., 2015; Waldbauer et al., 2017
Moisture Content (Dried)	4-10	Rana et al., 2015; Waldbauer et al., 2017
Total Dietary Fiber	27-82	Waldbauer et al., 2017; Antonic et al., 2020
Fructose	18-31	Queji et al., 2010
Glucose	2.5-12.4	Queji et al., 2010
Sucrose	3.4-24	Queji et al., 2010
Lipids and Protein	< 5	Skinner et al., 2018
Ash	0.38 -1.6	Kauser et al., 2024
Pectin	3.5 - 14.32	Antonic et al., 2020
Malic acid	0.05 - 3.28 %	Antonic et al., 2020
Total phenolics	0.17 - 0.99	Antonic et al., 2020

Apple seeds also contain amygdalin, a cyanogenic glycoside. The amygdalin content in seeds ranges from 1 to 4 mg/g (Bartkiene et al., 2019). When amygdalin is broken down by β-glucosidase, it can produce cyanide. As seeds are part of apple pomace, cyanide toxicity has been pointed as a concern in utilization. However, because hydrogen cyanide has a boiling point of 26°C, it easily volatilizes during thermal processing (Martău et al., 2021).

The apple source, genetics, pressing conditions, microbial fermentation and post-processing conditions (drying temperature in particular) have been reported as major factors affecting final composition of apple pomace (Bhushan et al., 2008, Skinner et al., 2018, Zhang et al., 2021). Furthermore, the quantity and type of polyphenols varies considerably even from batch to batch of the same apple variety (Skinner et al., 2018).

Antibacterial Properties

The antimicrobial properties of apple pomace are mainly investigated in relation to derived extracts (Bruna et al., 2024, Kauser et al., 2024), and not the substrate itself. The undried apple pomace can be a substrate for microorganisms to thrive, although antifungal properties have been assigned to some apple pomace streams (Kauser et al., 2024). Apple pomace extracts have been shown to inhibit *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis,* (Zambrano et al., 2019, Arraibi et al., 2021, Bruna et al., 2024).

Specific phytochemicals from apple pomace have been found to be antimicrobial. For instance, ursolic acid has demonstrated activity against Gram positive bacteria (Cargnin and Gnoatto, 2017). Phloridzin and phloretin extracted from apple pomace have also inhibited the growth of *S. aureus* and *E. coli* (Zhang et al., 2016). Notwithstanding, among the functional

properties of apple polyphenols, antioxidant activity receives most attention (Skinner et al., 2018). However, extracting and purifying specific compounds from apple pomace is still expensive and emerging as technology (Bruna et al., 2024).

Apple Pomace Against Pig Postweaning Diarrhea

Apple pomace has been sparingly investigated in piglet diets, showing various health benefits, but no studies have used infection challenge models (**Table 1.3**). In the studies by Sehm et al. (2006, 2007), inclusion of 35 g/kg feed of apple pomace in weaner diets increased villus length and decreased GALT activation in Peyer's patches, while postweaning growth was unaffected. The researchers also observed an upregulation of apoptotic genes in the liver and a downregulation of NFkB and TNFα genes in the stomach, suggesting a potential anti-inflammatory action (Sehm et al., 2006).

Table 1.3 Summary of studies on the dietary inclusion of apple pomace in weaning piglets. ↗/↘: increased / decreased compared to untreated control.

inclusion	Main Findings	Reference
Dry Apple pomace, 3.5%	Inflammatory and apoptotic genes in liver ↗, Inflammatory and apoptotic in stomach and jejunum ↘, villus length ↗, GALT activation in Peyer's patches ↘, no change in growth, digestibility or organ weight.	(Sehm et al., 2006, Sehm et al., 2007)
Fermented apple pomace, 4%, 6%, 8%	Apparent digestibility of CP ↗, IgA, IgG, IL-6 in serum ↗, intestinal coliforms ↘, Bacillus and Lactobacillus ↗, villus height ↗, crypt depth ↘	(Wang GuoJun et al., 2017)
Dry Apple pomace, 2%, 4%	ADG ↗ (week 3 postweaning), FCR ↗, tendency to fecal consistency and pathogen counts ↗, influenced microbiota richness and profile	(Dufourny et al., 2021)
Dry Apple pomace, In vitro culture medium	Propionate molar ratio ↗, butyrate molar ratio ↘, increased Prevotella and Akkermansia	(Dufourny et al., 2022)
Fermented apple pomace, 5%	ADG ↗, albumin and superoxide dismutase ↗, aspartate aminotransferase and malondialdehyde ↘, Lactobacillus abundance ↗, Ruminococcus ↘	(Ao et al., 2022)

More recently, supplementation of piglet diets with 20 and 40 g/kg of apple pomace improved postweaning growth performance and energy utilization, particularly at the higher inclusion level (Dufourny et al., 2021). However, it tended to decrease fecal consistency and increase pathogen counts, which the authors speculated might be related to the fiber profile of apple pomace. However, the reported carbohydrate profile of the diets does not show major differences, indicating another underlying reason. Furthermore, apple pomace increased microbial diversity, though it did not influence short chain fatty acids (**SCFA**) concentrations (Dufourny et al., 2021). Dufourny et al. (2019) investigated apple pomace using an in vitro porcine GIT model; apple pomace increased propionate and decreased butyrate concentrations and resulted in higher abundances of *Prevotella* and *Akkermansia.*

Fermented apple pomace has also been investigated as an alternative to drying and for increasing nutritional value. In a study by Wang GuoJun et al. (2017), supplementation with 60 g/kg of fermented apple pomace in piglet diets improved serum immune markers such as IgA,

IgG, and IL-6, reduced intestinal coliform counts, and increased beneficial bacteria populations like *Bacillus* and *Lactobacillus*. Ao et al. (2022) also found that fermented apple pomace (50 g/kg diet) enhanced growth performance, plasma antioxidant indicators, and positively modulated the gut microbiota. Apple pomace, in both dry and fermented forms, shows promise as a dietary supplement in piglet diets by improving growth performance, enhancing immune markers, and modulating gut microbiota. However, further research is needed to fully understand its potential in preventing postweaning diarrhea, particularly under infection challenge models.

Blackcurrant

Blackcurrants (*Ribes nigrum*) are the berries of a perennial shrub native to central and northern Europe and Asia (Allwood et al., 2019), currently cultivated across the temperate zones of Europe, Asia, New Zealand and recently North America (Allwood et al., 2019, Cortez and Gonzalez de Mejia, 2019). Blackcurrants are commonly used for producing juice, jams, and syrups; it has a distinct rich purple-black color given by the anthocyanin profile of the berries (Tabart et al., 2006). Blackcurrant intake has been attributed therapeutical properties due to the content of anthocyans, flavonols, phenolic acids and polyunsaturated fatty acids (Gopalan et al., 2012).

Blackcurrant products include blackcurrant powder, pomace, and encapsulates (**Figure 1.9**). Blackcurrant powder is usually produced from freeze dried fresh berries (Xue et al., 2022) and it has been used for food and nutraceutical applications (Mofasser Hossain et al., 2017). The blackcurrant pomace is the byproduct resulting from juice extraction, it is regarded as source of bioactive compounds and dietary fiber (Xue et al., 2022). The pomace can be further processed into powder or used for extraction of phytochemicals (Reißner et al., 2019).

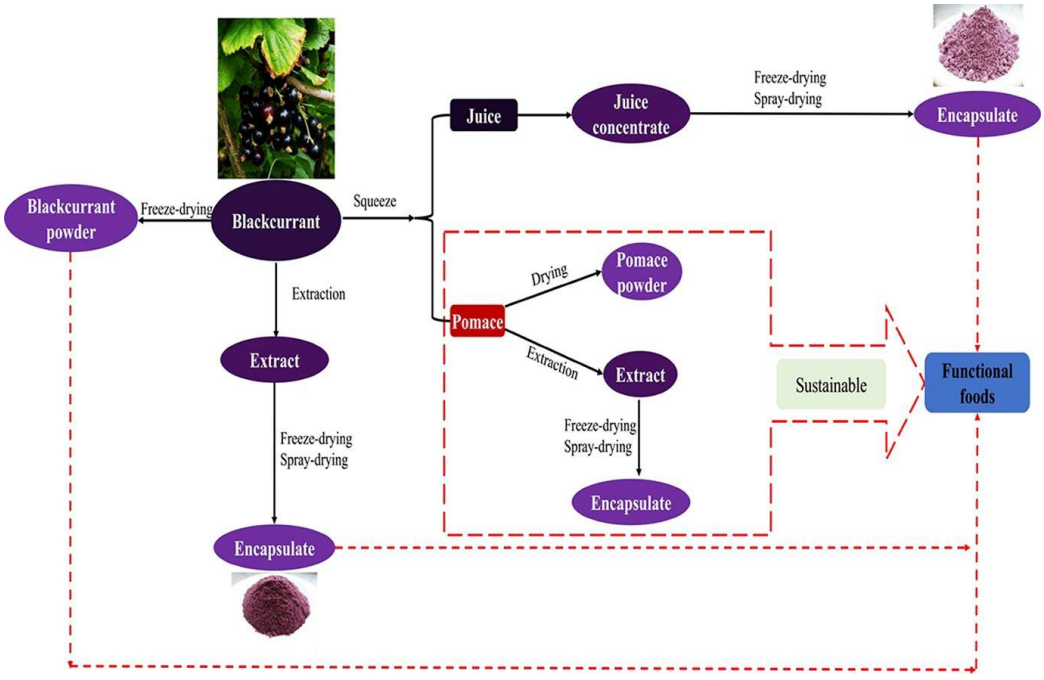


Figure 1.9 Processing of blackcurrant berries into various products. Extracted from Xue et al. (2022) (CC BY 4.0)

Blackcurrant berries have an approximate composition of ~78% moisture, 1.3% protein, 0.3% fat, 17.5% N free extract, 3% crude fiber and 0.3% crude ash (Jeong et al., 2012). Glucose

and fructose make up about 90% of the total non-structural sugars (~400mg/g DM) in the blackcurrant berries (Bordonaba and Terry, 2008). In blackcurrant powder (~2% moisture), the total dietary fiber composition has been reported to be ~42%, from which 22.5% is soluble and 19.5% is insoluble (Hui et al., 2021). The composition of blackcurrants can vary by several factors including genetic, agronomic and environmental factors (Tian et al., 2019).

Table 1.4 General composition of blackcurrant berries

Component	Value	Sources
Moisture	~78%	Jeong et al., 2012
Protein	1.3%	Jeong et al., 2012
Fat	0.3%	Jeong et al., 2012
Nitrogen-Free Extract	17.5%	Jeong et al., 2012
Total Dietary Fiber	~42% DM	Hui et al., 2021
Soluble Dietary Fiber	22.5 % DM	Hui et al., 2021
Insoluble Dietary Fiber	19.5% DM	Hui et al., 2021
Ash	0.3%	Jeong et al., 2012

Research on blackcurrant composition has primarily focused on the fruit's rich content of organic acids and phenolic compounds, revealing potential beneficial uses. For instance, citric acid, which comprises about 85% of the total organic acids (~160 mg/g DM) in blackcurrants, highlights the fruit's acidic profile (Bordonaba and Terry, 2008). Similarly, the total phenolic content ranges from ~ 60 to 300 ppm DM, with anthocyanins accounting for about 50% of this group, predominantly cyanidin and delphinidin derivatives (Slimestad and Solheim, 2002, Rubinskiene et al., 2005, Tian et al., 2019). Flavonols follow anthocyanins with concentrations ranging 18–60 mg/100 mg DM, from which myricetin glycosides are the dominants (Tian et al., 2019). Some phenolic acids are also found in blackcurrant berries from which caffeic and ferulic acid are the most common (Tian et al., 2019). The polyphenol content and profile varies by cultivar and growing conditions (Bordonaba and Terry, 2008, Zheng et al., 2012, Tian et al., 2019, Pott et al., 2023).

Antibacterial Properties

Research on blackcurrant products has revealed their significant potential in combating a range of pathogenic microbes. Blackcurrant displays antibacterial activity to Gram positive and Gram-negative pathogens. Blackcurrant juice and extracts have been shown to effectively suppress various bacteria including *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *E. coli* (Miladinović et al., 2014). Additionally, lyophilized blackcurrant powders have demonstrated inhibitory effects on *Salmonella enterica* and *S. aureus* (Puupponen-Pimiä et al., 2005). Polysaccharides from blackcurrant seeds decreased the adhesion of Helicobacter pylori to the gastrointestinal mucosa, showing anti-adhesive properties (Lengsfeld et al., 2004). These results collectively demonstrate that multiple components within blackcurrants possess the capability to inhibit a variety of bacteria effectively.

Organic acids and polyphenols in blackcurrants are believed to be responsible for the

observed antimicrobial activity (Puupponen-Pimiä et al., 2005, Raudsepp et al., 2019). These compounds exhibit a range of bioactive properties, including the ability to disrupt microbial cell walls and inhibit bacterial enzyme systems (Puupponen-Pimiä et al., 2005). Organic acids such as citric acid lower the pH of the environment, which can inhibit the growth of pathogens (Puupponen-Pimiä et al., 2005). Polyphenols, on the other hand, interact with bacterial proteins to destabilize cellular functions and prevent proliferation (Papuc et al., 2017). Together, these substances contribute to the broad-spectrum antimicrobial effects of blackcurrants, making them potentially valuable in preventing and treating infections.

Blackcurrant Against Pig Postweaning Diarrhea

Studies investigating the dietary inclusion of blackcurrant in piglets are limited, and available research primarily focuses on the metabolism of anthocyanins by piglets as a model for human nutrition. Wu et al. (2005) found that blackcurrant delphinidin and cyanidin are effectively absorbed in the gut and excreted intact in the urine. Additionally, Walton et al. (2006) reported that supplementation with blackcurrant powder significantly increased the antioxidant capacity in pig plasma, indicating enhanced systemic antioxidant status and potential anti-inflammatory effects. In support of this findings, Pieszka et al. (2017) found that dietary supplementation with blackcurrant pomace in fattening pigs tended to increase feed intake and increased the oxidative stability and vitamin E content in pork meat. Hence, these studies point at the potential of blackcurrant supplementation to support oxidative and inflammatory status of pigs.

Combination for Enhanced Activity

Recent research, including work from our group, has indicated that natural plant materials like wild garlic bulbs and lingonberries can reduce coliform populations in the GIT of piglets while preserving lactic acid bacteria, attributed to the allicin provided by wild garlic and the organic acids provided by the berries (Canibe et al., 2018). However, the limited availability and high cost of these plants prompt the exploration of more accessible alternatives.

Garlic, as a more industrially available allicin-containing material, can leverage its antibacterial properties. Additionally, integrating either apple pomace or blackcurrants into this combination could provide a viable alternative for utilizing these bioresources. When paired with apple pomace, garlic components may interact with the apple pomace dietary fibers and phenolics. For instance, Lahiri et al. (2021) reported the synergistic action of allicin and quercetin against *Bacillus cereus* by reducing virulence via biofilm-forming protein binding. The dietary fiber (e.g. from apple pomace or from garlic itself) in the diet and in the combination may potentially aid in the slow release of allicin and other phytochemicals, keeping its activity within the GIT over a longer period (Das et al., 2023).

Allicin has demonstrated significant potential to enhance the effectiveness of antibiotics through synergistic interactions (Choo et al., 2020). This property of allicin suggests that it might also act synergistically with other antibacterial agents, such as those found in apple pomace and blackcurrants. A similar mechanism has been suggested for the combination of essential oils with organic acids to target ETEC (He et al., 2022). Essential oils can alter the structure and function of bacterial cell membranes, leading to membrane swelling and increased permeability (Langeveld et al., 2014). This change in the bacterial membrane structure makes

the bacteria more vulnerable to other antimicrobial agents (He et al., 2022). By increasing cell membrane porosity, allicin might facilitate penetration of other antibacterial compounds into bacterial cells, potentially leading to synergistic action.

Furthermore, combining garlic with apple pomace or blackcurrants introduces a different set of synergistic effects. For instance, Lencova et al. (2022), reported enhanced antibacterial action of a garlic extract when combined with erythorbic acid (isoascorbic acid). Similarly, previous studies[9] conducted in our group, investigated the antibacterial effects of various plant materials at different concentrations, both alone and in combination, against ETEC F4 and F18 using stomach and small intestinal ileal content incubations in vitro (Højberg, Lauridsen et al., 2022, Canibe et al., 2022). The combination of ramsons (wild garlic) and acidic berries (lingonberries or redcurrants) demonstrated a more potent antibacterial effect than when used individually (Lauridsen et al., 2022). It has been suggested that the synergy likely results from the combined action of wild garlic's antibacterial components (including allicin) and the organic acids in the fruits, though the exact mechanism requires further investigation (Højberg, Canibe et al., 2022, Lauridsen et al., 2022).

Furthermore, a pilot in vivo study with piglets resulted in reduction in *Enterobacteriaceae* but not lactic acid bacteria in the gastrointestinal tract when fed a diet supplemented with 3% ramsons and 3% lingonberries (Canibe et al., 2018). These results highlighted the potential of combining allicin containing plants with fruits and berries resulting in more effective antibacterial properties. Furthermore, these early findings created a foundation for the current thesis which aimed to use this proof of concept with more widely available plant materials.

The interactions occurring from the combination of allicin containing garlic and fruits/berries could play a role in the generation of a hostile environment for ETEC to proliferate in the piglets GIT (**Figure 1.10**). The reduced gastric acid secretion in weaned piglets leads to a higher stomach pH compared to sow-reared piglets, making them more susceptible to enteric infections and affecting their ability to digest nutrients (Heo et al., 2013). The Acid Binding Capacity (**ABC**) of a diet, which reflects its resistance to a low pH, plays a crucial role in this context (Lawlor et al., 2020). Diets with a high ABC can decrease the digestibility of dry matter and proteins, hinder growth performance, and increase the release of harmful substances like amine and ammonia, potentially causing diarrhea (Lawlor et al., 2005, Wang et al., 2023). Therefore, incorporating apple pomace or blackcurrant could lower ABC in the piglet feed thereby aiding gastric acidity. This, in conjunction with antibacterial properties of phytochemicals present in the plant materials could potentially improve efficacy against ETEC.

[9] MAFFRA I project (Antibacterial Plants Against Diarrhea in Pig Herds), twelve plant species were identified for their activity against pathogenic *E. coli*, as described in *GENERAL INTRODUCTION* section.

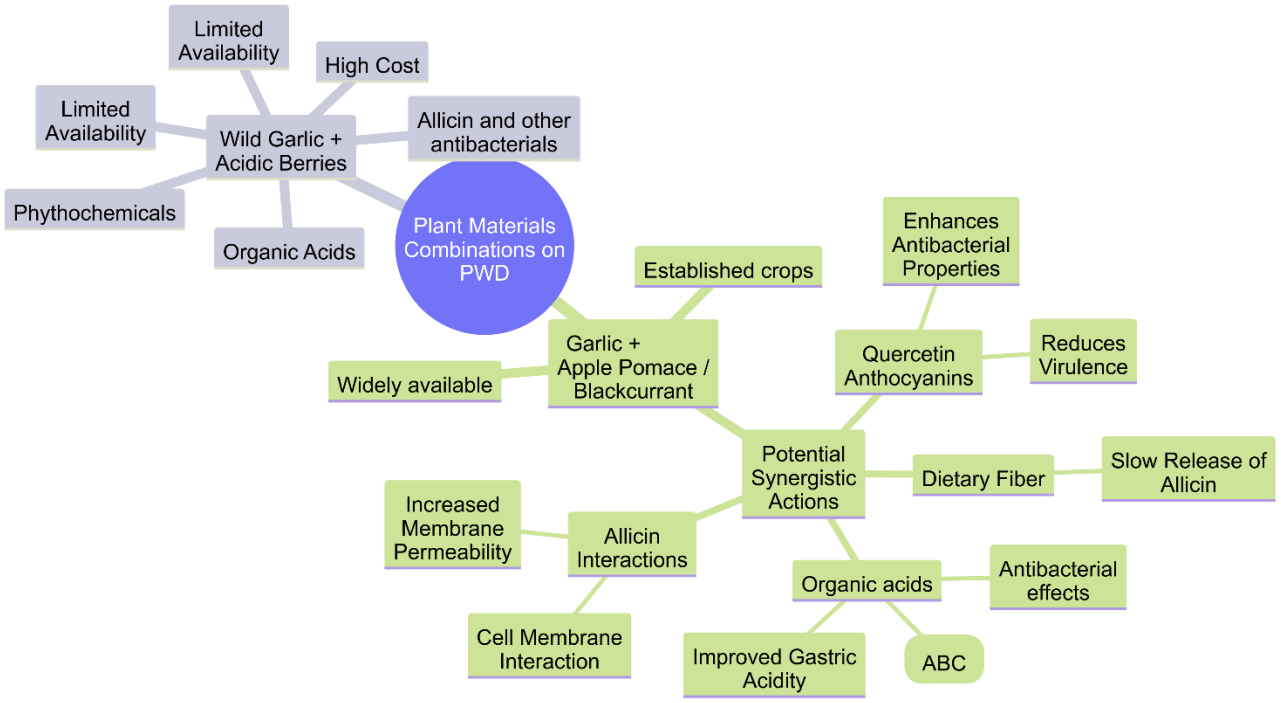


Figure 1.10 Using plant combinations to target *ETEC* and prevention of PWD.Potential mechanisms for synergistic antibacterial effects of combining garlic with apple pomace or blackcurrants in weaning piglet diets to target ETEC.

1.3.2 Plant Natural Products Targeting *Clostridium perfringens*

Lauric Oils and Medium Chain Fatty Acids

Lauric oils are oils and fats in which lauric acid (C12:0) is the main fatty acid, comprising about 50%, compared to less than 1% in most other vegetable oils (Amri, 2011). The most industrially important lauric oils are coconut and palm kernel oil. Other lauric oils include babassu, tukum, murumuru, ouricuri, which are locally sourced and produced in small quantities (Amri, 2011). Both coconut oil and palm kernel oils have prominent levels of medium- and long-chain saturated fatty acids (Amri, 2011). These plant oils are often referred to as medium chain triglyceride oils as well (Dayrit, 2014). Coconut oil is more saturated than palm kernel oil, with iodine values of 6-10 and 14-21, respectively (Amri, 2011). They have a melting point of 25-27 °C and are more resistant to oxidative rancidity than other oils (Kinderlerer, 1994).

Coconut and Palm kernel oils are regarded as rich and natural sources of medium chain fatty acids (**MCFA**). The MCFA are not only a source of energy, they are reported to improve the intestinal epithelial structure and are characterized by strong antibacterial activity (Zentek et al., 2011, Hanczakowska, 2017). Naturally occurring MCFAs are fatty acids with 6 to 12 carbons and one carboxylic group, namely, caproic (C6:0), caprylic (C8:0), capric (C10:0), and lauric (C12:0) acids. Furthermore, MCFAs have higher energy density than short chain fatty acids (**SCFAs**, C2–C5) (O-Thong et al., 2020). In the animal feed industry and technology, they are often grouped together with other organic acids as a category for feed additives (Hanczakowska, 2017). The production of MCFA comes primarily from the palm refining industry, but they can also be produced from animal fats, petrochemically and via microbial fermentation (Ho Ahn et al., 2023).

The composition of coconut and palm kernel oils is similar, both oils are rich in lauric acid

but differ in their levels of caprylic, capric and oleic acids (**Table 1.5**). Crude coconut oil primarily contains lauric acid, with significant amounts of caprylic, capric, myristic, and palmitic acids. Similarly, crude palm kernel oil is also rich in lauric acid, but features notable levels of oleic acid (Amri, 2011). The fatty acid profile of both oils can be affected by the environmental and growing conditions (Young, 1983, Montoya et al., 2014).

Table 1.5 Fatty acids content of coconut, and palm kernel oils (Amri, 2011).

Fatty Acid	Coconut Oil (%)	Palm Kernel Oil (%)
Caprylic Acid (C8:0)	5-10	3-6
Capric Acid (C10:0)	5-8	3-5
Lauric Acid (C12:0)	45-55	45-55
Myristic Acid (C14:0)	17-21	14-18
Palmitic Acid (C16:0)	8-10	7-10
Oleic Acid (C18:1)	5-10	12-19
Other Fatty Acids	~5-7%	~5-7%

Plant oils primarily consist of triglycerides, which are composed of fatty acids and glycerol. These oils are industrially hydrolyzed into their constituent parts through a process called fat or oil splitting (Young, 1983). Fatty acids from palm kernel oil and coconut oil are used in various industries, including food, cosmetics, animal feed, pharmaceuticals, and biodiesel (Amri, 2011). Since they typically occur as triglycerides, their hydrolysis into free fatty acids is essential for showing their antibacterial properties (Shilling et al., 2013).

Antibacterial Properties

Hydrolyzed forms of lauric oils, such as free fatty acids and monoacylglycerols, have demonstrated antibacterial activity against various bacterial species (Thormar, 2010). Extensive research has shown that lauric acid is particularly effective against Gram-positive bacteria (Kabara et al., 1972), followed by other MCFA. Notably, early studies also indicated that monocaprin (the monoglyceride of C10:0) and monolaurin (the monoglyceride of C12:0) exhibit greater antibacterial activity than their respective free fatty acid forms (Kabara et al., 1972). These findings suggest that the structural forms of fatty acids, whether free or bonded, significantly influence their antimicrobial properties.

The antibacterial properties of lauric acid against clostridial pathogens have been recognized for decades (Galbraith et al., 1971). Subsequent research has specifically investigated lauric acid as an antibacterial agent targeting clostridial pathogens (Shilling et al., 2013, Yang et al., 2018, Yang et al., 2019b). Skřivanová et al. (2005) observed strong inhibition of two *Clostridium perfringens* strains by lauric acid in a study that evaluated the effects of fatty acids ranging from C2 to C18. This study also found that myristic, capric, oleic, and caprylic acids inhibited *C. perfringens*, at higher concentrations to that of lauric acid, while linoleic acid (C18:2) was effective against only one strain (Skřivanová et al., 2005). The effectiveness of lauric acid against *C. perfringens* has been consistently reported in other studies as well (Timbermont et al., 2010, Zeitz et al., 2015, Matsue et al., 2019, Gomez-Osorio et al., 2021). Moreover, lauric acid

exhibits low antimicrobial activity against gut commensal lactic acid bacteria such as lactobacilli, suggesting its potential for selective antibacterial applications without significantly disrupting beneficial microbiota (Matsue et al., 2019).

The antibacterial mode of action of free fatty acids is not fully understood, but they commonly target bacterial cell membranes (Thormar, 2010, Yoon et al., 2018). The amphipathic[10] nature of fatty acids facilitates interactions with these membranes (Figure 7), where they can create temporary or permanent pores of varying sizes (Desbois and Smith, 2010). The membrane-destabilizing activity increases cell permeability disrupts intracellular systems, and ultimately leads to cell lysis (Thormar, 2010). Additionally, free fatty acids at high concentrations function as detergents, disturbing the membranes to the extent that proteins or segments of the lipid bilayer are dislodged, further compromising the integrity and functionality of bacterial cells (Desbois and Smith, 2010). This multifaceted disruption of cellular structures underscores the capability of free fatty acids as effective antibacterial agents.

Exposure to lauric acid has been shown to cause significant damage to bacterial cells, including abnormal cell morphology, disrupted membranes, and leakage of cytoplasmic contents (Yoon et al., 2015, Yang et al., 2018). It is proposed that lauric acid penetrates bacterial cells, altering their intracellular pH. This alteration disrupts critical cellular functions such as enzymes, nutrient transport systems, and ATP-driven pumps, ultimately leading to cell death (Freese et al., 1973, Khoramnia et al., 2013). Notably, in Skřivanová et al. (2005), exposure of *C. perfringens* to lauric acid resulted in the separation of inner and outer membranes and complete cytoplasmic disorganization, although the cell wall structure and cell size remained unchanged (**Figure 1.11**). These observations underline the distinct mechanisms of lauric acid compared to other fatty acids, demonstrating its unique ability to disrupt bacterial systems beyond membrane disruptions.

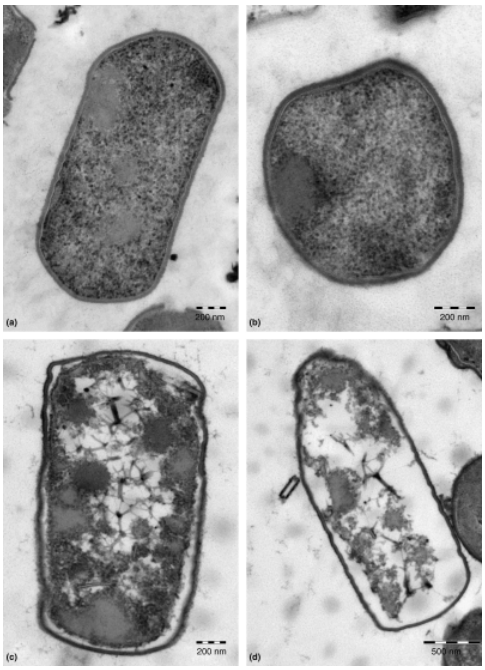


Figure 1.11 Effect of lauric acid on *C. perfringens* via transmission electron microscopy. Top pictures (a, b) show bacterial cells growing in control media. While bottom pictures show bacterial cells exposed to lauric acid (1 mg/ml) for 30 minutes. Extracted from: Skřivanová et al. (2005).

[10] The carboxyl group in fatty acids is hydrophilic and becomes ionized when dissolved in water. Meanwhile, the carbon chain and terminal methyl group are hydrophobic. (Yoon et al., 2018).

Essential Oils

Essential oils are complex mixtures of volatile compounds isolated by pressing or distillation from plant materials (Thormar, 2010). Essential oils are formed by mixture of several compounds, predominantly terpenes, depending on the source phenylpropanoids, organosulfurs, nitrogen containing compounds and others. Nevertheless, most of the composition is generally given by 1- 3 components that make up 85% of the oils (Zhai et al., 2018). For instance, thymol and carvacrol, which can form about 80% of oregano essential oil. Whereas eugenol in clove essential oil represents 70 to 90% of the oil (Hu et al., 2018). These major components determine the biological properties of essential oils. Terpenes, terpenoids and other aromatic constituents can be found in essential oils at different concentrations (Figure 1.12). Essential oils from *Allium* plants (e.g., onions, leek, garlic) contain mostly volatile thiosulfinates (Ross et al., 2001).

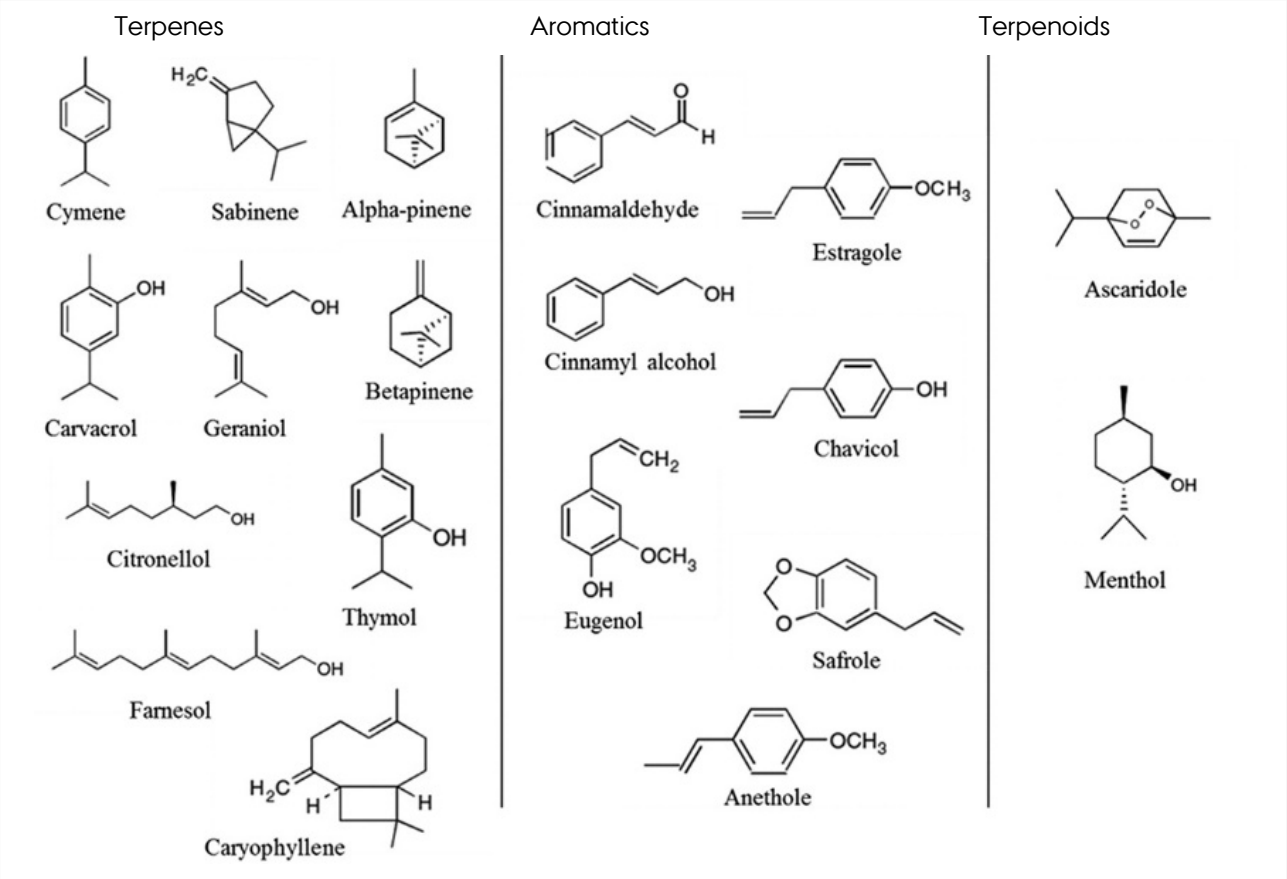


Figure 1.12 Chemical structures of selected essential oil components. Adapted from Baptista-Silva et al. (2020).

Antibacterial Properties

Essential oils, combinations, and single components have displayed a wide range of antimicrobial activities (Rossi et al., 2020). For instance, the essential oils of rosemary, sage, oregano, thyme, clove, cinnamon, and lemon grass have been effective against the enteropathogenic strains *E. coli*, *Salmonella spp*. and *C. perfringens* (Franz et al., 2010, Chouhan et al., 2017). Single components of essential oils, such as carvacrol, thymol, cinnamaldehyde, and eugenol, exhibit significant antimicrobial properties against *E. coli*, *Campylobacter jejuni*, *Staphylococcus aureus*, and *Salmonella spp* (Zhai et al., 2018).

Additive, antagonistic and synergistic action between components of essential oils have

been reported (Bassolé and Juliani, 2012). For instance, thymol, carvacrol, eugenol have demonstrated synergistic or additive effects (Bassolé and Juliani, 2012). Essential oil combinations may also display interactions. For example, Ahmed Khan and van Vuuren (2021) investigated an extensive list of essential oils against *C. perfringens* and found that among 196 combinations, only 9 proved synergistic action, while 77 resulted in antagonistic interactions. In addition, ingested essential oils can interact with the components of the diet affecting their antimicrobial efficacy (Zhang et al., 2014).

Essential oils have been extensively used to control ANE in broilers. As summarized by Abd El-Hack et al. (2022) and Diaz Carrasco et al. (2016), essential oils of oregano, thyme, ginger oil, carvacrol, peppermint oil and multiple blends (including for example clove, oregano, cinnamon, rosemary, eucalyptus, anis, among others) have been shown to successfully prevent *C. perfringens* and improve broiler growth efficiency. Whereas research on single components has shown cinnamaldehyde, eugenol, thymol and carvacrol, and their combinations as effective against ANE (Du et al., 2015, Du et al., 2016, Reis et al., 2018, Kumar et al., 2021).

The antibacterial mode of action of essential oils can act on single or multiple cellular targets. For example, terpenes interact with bacterial cell membranes by binding phospholipids affecting the structure and function of the membrane (e.g., proton pumps, ATP regulation) (Hyldgaard et al., 2012). Eugenol, activity by permeabilizing cell membranes and interacting with proteins, causing increased transport of potassium and ATP out of cells and inhibiting various enzymes, including ATPase (Hyldgaard et al., 2012). Cinnamaldehyde, disrupts microbial functions by cross-linking with DNA and proteins, inhibiting cytokinesis by binding to the FtsZ protein, and interfering with ATPase activity at sub-lethal concentrations, while also altering membrane lipid profiles and causing membrane depolarization and loss of integrity (Hyldgaard et al., 2012). Additionally, essential oils can interfere with membrane proteins and intracellular targets, leading to uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites, and disruption of DNA, RNA, protein, lipid, and polysaccharide synthesis (Rossi et al., 2020).

Increasing dosages of essential oils have been shown to alleviate intestinal lesions in chickens, even though *C. perfringens* populations remain unchanged (Du et al., 2016). This suggests that essential oils may prevent ANE through mechanisms other than direct antibacterial action. The luminal concentrations of essential oils are lower than their in vitro minimum inhibitory concentrations (**MIC**), indicating alternative modes of action (Du et al., 2016). For instance, quorum sensing inhibitors identified in several essential oils imply their influence on bacterial virulence development (Saeki et al., 2020). The Agr-Like quorum sensing system is critical for ANE pathogenesis (Yu et al., 2017). Additionally, essential oils supplementation enhances immunity in chickens. Specifically, essential oil compounds inhibit NF-κB and MAPK pathways, regulating pro-inflammatory cytokines and increasing intestinal integrity (Valdez et al., 2023). Therefore, essential oils may reduce bacterial virulence through quorum sensing inhibition and alleviate inflammation by modulating immune responses.

Recent studies incorporating essential oils (**Table 1.6**) have focused on investigating new essential oils (Coles et al., 2021), microencapsulation techniques to enhance stability and efficacy (Yang et al., 2016, Jin et al., 2022), and synergistic effects when combined with other bioactive compounds like lysozyme and organic acids (Du et al., 2021; Pham et al., 2020). These efforts aim to improve the antimicrobial, anti-inflammatory, and antioxidant properties of broilers facing ANE challenges.

Table 1.6 Recent controlled challenged studies investigating essential oils against necrotic enteritis in broiler chickens.

Essential Oil	Main Findings	Reference
Lippia origanoides	↘ lesions, ↘ morbidity, ↗ SOD	Coles et al., 2021
Origanum vulgare (microencapsulated)	↗ BWG, ↘ FI drop, ↘ TNF-alpha	Jin et al., 2022
Thymol, Carvacrol (+lysozyme)	↘ mortality, ↗ villus height, no effect on *C. perfringens*, essential oils ↘ lesions but no in combination with lysozyme.	Du and Guo, 2021
Eugenol (+ Garlic tincture)	↗ FCR, ↗ livability, ↘ lesions	Kumar et al., 2022
Carvacrol, thyme (Encapsulated + Caproic, benzoic, and butyric acid)	↗ FCR, ↗ villus height, ↘ *C. perfringens*, ↗ gene expressions (claudin-1, GLP-2, IGF-2), ↗ Lactobacillus, Enterococcus	Pham et al., 2020
Thymol, Carvacrol (+ *Eimeria* vaccination)	↘ lesions, ↗ performance	van Eerden et al., 2022

Combination for Enhanced Activity

Combining lauric oils with essential oils may offer synergistic interactions that enhance their antibacterial efficacy. Lauric acid and other medium-chain fatty acids (MCFAs) in lauric oils, when used in conjunction with essential oils—containing a complex mixture of bioactive compounds such as terpenes, phenylpropanoids, and organosulfurs—may result in improved efficacy. Both lauric acid and essential oils disrupt bacterial cell membranes through different mechanisms and also target intracellular components (Thormar, 2010, Langeveld et al., 2014). This dual action can lead to more extensive and rapid bacterial cell death, making the combination particularly effective.

Furthermore, both essential oils MCFAs have been reported to work synergistically with conventional antimicrobials (Langeveld et al., 2014, Ghany et al., 2024). Additionally, recent studies have shown that combining essential oils (e.g., thyme, clove, oregano, cinnamon) with organic acids (e.g., formic, citric, lactic, propionic) effectively enhances antibacterial efficacy in broilers, improving growth performance, gut health, nutrient digestibility, and reducing lesions caused by pathogens like *C. perfringens* (**Table 1.7**). The high hydrophobicity of essential oils is thought to alter cell membrane permeability, allowing organic acids to penetrate the cytoplasm more effectively. Once inside the cells, these organic acids, in their undissociated form, lower the internal pH and disrupt bacterial metabolism (Stefanello et al., 2020).

The combination of lauric acid, other MCFAs, and essential oils offers another promising strategy for enhancing antibacterial efficacy. For instance, Abdelli et al. (2020) explored the use of microencapsulated blends of organic acids, including malic, fumaric, and calcium butyrate, combined with cinnamaldehyde, carvacrol, and thymol. While these studies did not evaluate the effects of individual components, they demonstrated significant improvements in growth performance and gut health in broilers under ANE challenge. Nevertheless, as mentioned before, antagonistic activities has been observed when combining essential oils against *C.*

perfringens (Ahmed Khan and van Vuuren, 2021), thus rigorous screening is necessary to identify possible interactions when combining essential oils and MCFA.

Table 1.7 Summary of studies on the combined effects of essential oils and organic acids on antibacterial efficacy in broilers.

Essential Oil	Organic Acid	Main Findings	Reference
Cinnamon, Clove, Thyme	Propionic, Citric, Butyric	Performance ↗, Intestinal morphology ↗, Cecal microflora ↗, Jejunal enzyme activity ↗	Gao et al., 2019b
Thyme, Clove, Oregano	Formic, Citric, Lactic	Villus height ↗, Crypt depth ↗, Gut health ↗, Morbidity ↘, Lesions ↘	Pham et al., 2020
Cinnamaldehyde, Carvacrol, Thymol	Malic, Fumaric, Calcium Butyrate, Citric acid Capric, Caprylic, Caproic, Lauric	Growth ↗, Villus height ↗, Crypt depth ↗, *Ruminococcaceae* ↗, *Lachnospiraceae* ↗, *Enterobacteriaceae*↘, *C. perfringens* ↘	Abdelli et al., 2020
Cinnamon, Clove, Thyme	Formic, Citric, Lactic	Growth performance ↗, Nutrient digestibility ↗, CLDN1 and OCLN ↗	Stefanello et al., 2020
Thyme, Clove, Oregano	Formic, Citric, Lactic	Growth performance ↗, Lesions ↘, Villus height ↗, *C. perfringens* ↘	Pham et al., 2022

1.4 CHALLENGES AND LIMITATIONS OF USING ANTIBACTERIAL PLANT COMBINATIONS TO TARGET GASTROINTESTINAL PATHOGENS

1.4.1 Use of Plant Materials

While the use of antibacterial plant combinations shows promise in targeting gastrointestinal pathogens for prevention of PWD and ANE, some challenges and limitations must be considered. One significant challenge is the variability in plant composition. The phytochemical composition of plant materials can vary significantly due to factors such as growing conditions, harvest time, and post-harvest processing (Martins et al., 2016, Kuete and Kuete, 2023). This variability can affect the consistency and efficacy of plant-based treatments. For instance, essential oils (Figueiredo et al., 2008) , garlic (Hirata et al., 2016), blackcurrants(Tian et al., 2019) and lauric oils (Amri, 2011)may have differing concentrations of active compounds depending on the variety, geographical origin and climate conditions where the plants were cultivated. This inconsistency can lead to variations in treatment outcomes and inconsistency in the efficacy across different batches of plant materials. Therefore, addressing the variability in plant composition is crucial for optimizing the use of plant-based antimicrobials in disease prevention. Efforts on standardization suggest titration on principal bioactive compounds as critical measure for antibacterial efficacy testing (Fankam and Kuete, 2023).

While many plant-derived compounds are recognized as safe, their long-term effects and potential toxicity at higher doses need thorough evaluation. For instance, some essential oils and plant extracts can cause adverse reactions, such as allergies or gastrointestinal irritation, particularly when used in high concentrations or over extended periods (Álvarez-Martínez et al., 2021). Therefore, it is crucial to conduct comprehensive safety assessments to ensure their safe use in animal feed. Furthermore, regulatory approval process for feed additives can be lengthy

and complex, posing challenges for their commercialization. Ensuring compliance with regulatory standards for safety, efficacy, and quality control can be resource-intensive, potentially limiting the accessibility and affordability of these products (Pandey et al., 2019). Hence, investigating minimally processed plant materials that provide functional properties is an advantage for further utilization.

1.4.2 Challenges in Assessing Interactions in Antibacterial Combinations

Interactions between plant phytochemicals or plant combinations can be classified as antagonistic, additive/non-interactive, or synergistic. The enhanced effectiveness of plant blends, compared to individual products, is often attributed to the synergistic effects of the mixture of bioactive constituents and their byproducts (Wagner and Ulrich-Merzenich, 2009). While the use of plant combinations has traditionally been preferred over isolated phytochemicals, this practice complicates the determination of their mode of action due to the possibility of complex interactions (Rossi et al., 2020). Indeed, most available data on the use of plant materials as non-antibiotic feed additives in livestock comes from strategies involving combinations of different plants, with little mechanistic insights (Windisch et al., 2008, Rossi et al., 2020). Furthermore, "mono-plant" materials may contain several bioactive substances thus interactions are likely to occur in single plant extracts as well (Wagner and Ulrich-Merzenich, 2009). In several instances single compounds lose their effectiveness when used isolated (Canibe et al., 2022).

Studying synergy and antagonism among plant-derived bioactive compounds is difficult due to the complexity of mixtures and the need for advanced methods to identify interactions. To accurately assess the synergistic effects of plant combinations in vitro, researchers employ various experimental designs. These include checkerboard assays, time-kill curves, and response surface modeling (Wagner and Ulrich-Merzenich, 2009, Vaou et al., 2022). Recent developments in metabolomics and high-throughput chemical screening promised an advanced tool for identifying synergistic and antagonistic interactions among bioactive compounds in plant materials (Vaou et al., 2022).

1.4.3 Interactions Between Plants and Gut Microbiota

The interaction between antibacterial plant extracts and gut microbiota is a crucial factor in determining their effectiveness (Iqbal et al., 2020). The GIT is inhabited by diverse microbial communities that are essential for digesting feed and absorbing nutrients. The gut microbiota produces various metabolites, including short-chain fatty acids (SCFAs), secondary bile acids, biogenic amines, indolic and phenolic compounds (Vasquez et al., 2022). Ammonia, hydrogen sulfide, neurotransmitters and vitamins are also produced by different gut microbes playing different roles, in some instances positive but also detrimental, particularly when in excess (Vasquez et al., 2022). The composition and activity of the gut microbiome, and consequently the production of metabolites, vary across different sections of the gastrointestinal tract and are influenced by factors such as age and diet (Vasquez et al., 2022). Interactions of the GIT microbiota with the host and the ingested feed are therefore warranted and often overlook (Danneskiold-Samsøe et al., 2019, Forgie et al., 2019, Patil et al., 2020, Sonnert et al., 2024).

Besides the ability to selectively modulate the composition og GIT microbiota, microbial interactions can influence the antibacterial activity of plant extracts. For example, gut bacteria

metabolize polyphenols into simpler, more bioactive compounds, enhancing their effectiveness (Iqbal et al., 2020). Some *lactobacillus* species can metabolize plant glycosylated polyphenols into aglycones, which increases their bioavailability (Theilmann et al., 2017).

1.5 SUMMARY AND IMPLICATIONS

The global shift away from antibiotics in livestock has posed significant challenges for the swine and poultry industries. The restriction of traditional antimicrobials, without effective alternatives, has led to increased infections and a rise in therapeutic antibiotic use. Despite over two decades of research yielding significant advancements, optimal antimicrobial strategies that ensure animal health, productivity and welfare are still needed.

Piglets and broiler chickens are particularly vulnerable to gastrointestinal disorders caused by pathogenic bacteria. These disorders include PWD in piglets and ANE in broilers, primarily caused by ETEC and *C. perfringens* type G[11], respectively. Traditional control methods are being restricted due to concerns over AMR and environmental pollution. Finding effective solutions would significantly improve the health, welfare, and productivity of these animals, while strategically contributing to the global fight against AMR.

Sustainable alternatives, such as plant-based antibacterials, offer promising solutions for enhancing gastrointestinal health in piglets and chickens. Plant materials have demonstrated strong antibacterial properties and are increasingly recognized as viable solutions. Furthermore, leveraging synergistic actions by combining different plant materials can enhance antimicrobial efficacy. Promising strategies include combining garlic with apple pomace or blackcurrant to target ETEC, and the use of lauric oils and essential oils to target *netB*+ *C. perfringens*. This approach not only meets consumer demands for natural and safe products but also supports the reduction of antibiotic use, aiding in the global effort to combat AMR.

Advanced screening methods for antibacterial efficacy, including both in vitro and in vivo testing, provide a comprehensive understanding of the interactions between plants, phytochemicals, diets, microbiota, pathogens, and hosts. This knowledge is crucial for developing effective solutions that can be reliably applied in commercial settings.

The aim of this thesis is to assess the efficacy of antibacterial plant combinations for the prevention of PWD and ANE by targeting gastrointestinal pathogens. A robust framework has been used to consider the characteristics of the plants, animals, and gut microbiota. The ultimate goal is to contribute to the balance between productivity, animal welfare, and sustainability in livestock systems, while addressing the public health challenges posed by AMR.

[11] The rest of the text will favor the use of *netB*+ *C. perfringens* (as opposed to type G *C. perfringens*), as it remains the most commonly used in the literature.

Chapter 2: METHODOLOGY

2.1 INTRODUCTION

The prevalence of gastrointestinal diseases in young monogastric animals, such as PWD in pigs and ANE in chickens, presents significant challenges to the livestock industry. Both diseases are multifactorial and involve identified bacterial agents. However, these diseases also result from complex interactions between the host, pathogens, and the environment. This multifaceted nature makes the investigation and development of effective solutions a challenging task. Understanding these intricate host-pathogen-environment interactions is crucial for devising prevention or management strategies.

Traditional germ theory, which posits that pathogens are the primary cause of infectious diseases, has been instrumental in developing preventive and therapeutic measures (Ross and Woodward, 2016). However, this microbe-centric view fails to account for nuances, such as why certain organisms are more susceptible than others or when some become asymptomatic carriers. In contrast, the "Full-Blown Host Theory," proposes that infectious diseases are primarily driven by the host's pre-existing immunodeficiencies, relegating the pathogens to a mere environmental trigger (Carlsson and Råberg, 2024). While this host-centric approach underscores the importance of host factors, it overlooks the evolved strategies of pathogens that actively manipulate host responses (Carlsson and Råberg, 2024). Indeed, the rise in studies on microbiota communities has provided new perspectives on the role of pathogens within microbial assemblages (O'Toole, 2024). Furthermore, the gut microbiota significantly contributes to host metabolism and health, influencing in part how the diet is digested and absorbed while modulating the immune status (Valdes et al., 2018). Therefore, the development of strategies to target gut pathogens and prevent diseases must incorporate a comprehensive view of the problem involving aspects of host, diet and microbes and their interactions to address diseases with complex pathogenesis such as PWD and ANE.

The studies in this thesis aim to provide a comprehensive understanding of the effect of using antibacterial plant material for the prevention or treatment of PWD in pigs and ANE in chickens. The following sections detail the rationale behind the experimental design, sample collection, diagnostic endpoints and analytical techniques employed in this research. The text cover the selection and preparation of plant materials for antibacterial testing, methods for assessing their antibacterial efficacy both in vitro and in vivo, and measurements of their effects on gut microbiota and host responses with particular focus on pig PWD and chicken ANE.

2.2 SELECTION OF PLANT MATERIALS

The process of selecting and processing plants for antibacterial testing involves several critical steps (**Figure 2.1**). The first selection of plant materials is influenced by previous reports of antibacterial effects or screening studies, often based on general properties or anecdotal evidence (Kuete and Kuete, 2023). Cultivation and collection involve choosing the plant variety, employing proper cultivation methods, and determining the optimal timing and conditions (Kuete and Kuete, 2023). The next phase involves processing the plants for antibacterial

applications. This step includes optimizing drying or extraction methods to enhance antibacterial effects or prevent the loss of properties (Kuete and Kuete, 2023). Identifying and characterizing active substances is critical for reporting and quality assurance, requiring extraction techniques like chromatography and characterization methods such as spectrometry (Ma and Qi, 2021). Finally, quality control involves the assessment of organoleptic, morphological properties, physicochemical properties, chromatographic profile, and toxicity (Kuete and Kuete, 2023).

Furthermore, the limitations imposed subsequent utilization need to be taken into account. For instance, if the plant materials are to be incorporated into the diet of the animals, the processing steps involved in the feed may affect their efficacy. Various feed processing steps can influence the characteristics of the plant materials, including grinding (particle size reduction), mash conditioning (temperature, moisture content, retention time), pelleting or extrusion (temperature, pressure, shearing, moisture content, retention time), and cooling/drying systems (der Poel et al., 2020). Recognizing and controlling these factors is essential to ensure the efficacy and consistency of the final products.

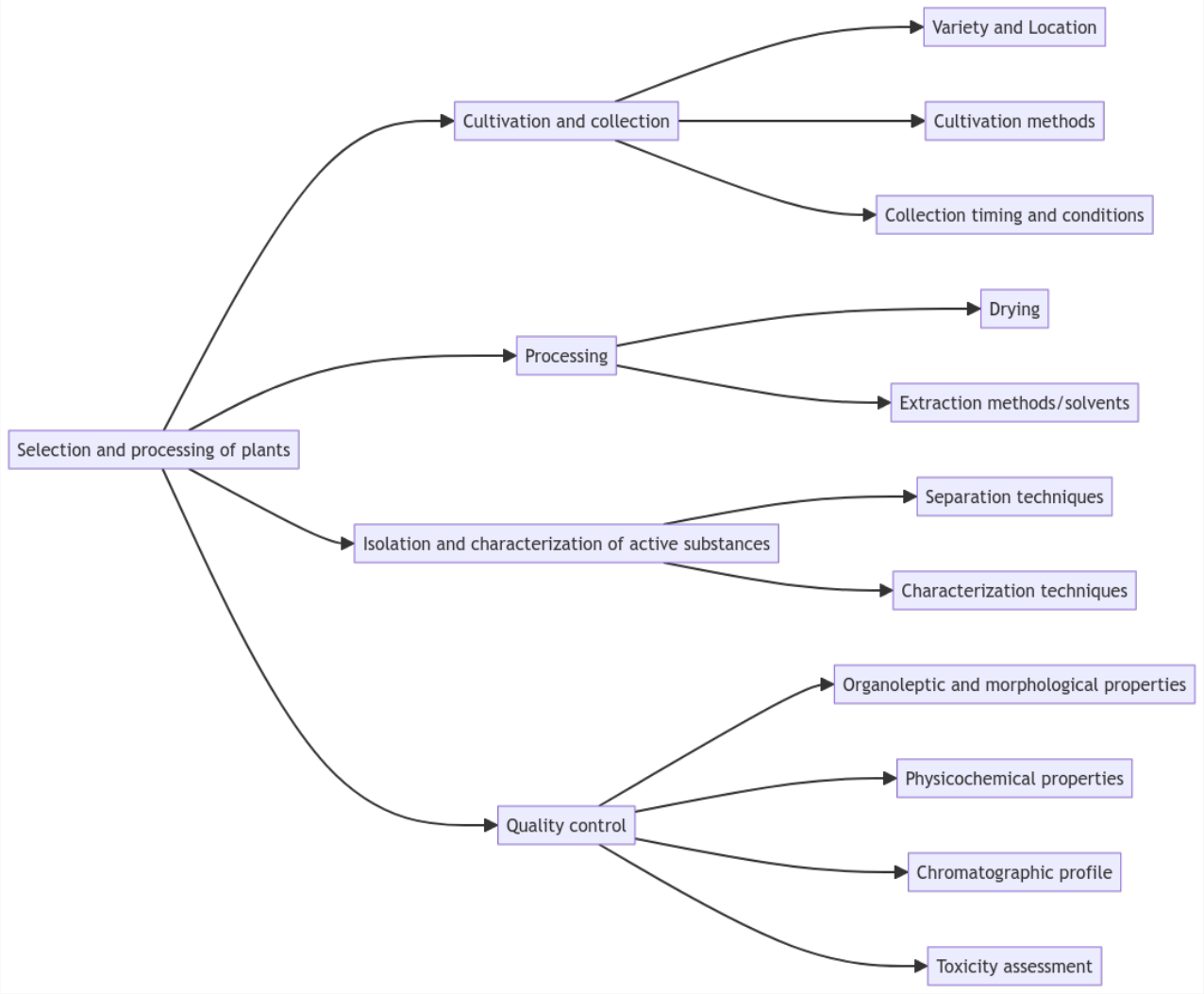


Figure 2.1 Considerations for choice and preparation of plant materials. Steps involved in the quality assurance for antibacterial applications. Based on: Kuete and Kuete (2023).

In this thesis

Study-I and Study-II: Garlic, apple pomace and blackcurrants

The plants selected for the study were chosen based on previous in vitro and pilot studies conducted in our research group, with the primary decision criteria being the selection of an allicin-containing plants and sources of organic acids, based on earlier tests using wild garlic and acidic berries. Garlic was chosen as the source of allicin, and blackcurrant berries were selected as sources of organic acids and polyphenols. Additionally, apple pomace was included for its phytochemical content and to provide a sustainable use for this currently underutilized material. All plant materials were organic to align with their application in organic diets.

Garlic was selected based on screening several varieties for optimal allicin content, the main active substance. Careful processing was conducted to maintain allicin's potency, avoiding the denaturation of alliinase and premature allicin formation. The processing of apple pomace also took care to prevent cyanide formation through thermal inactivation. All materials were stored frozen until use to prevent decay or changes in their physicochemical characteristics.

Quality control measures assessed the final allicin concentration in garlic powder and used pH as a proxy for organic acid concentrations in the apple pomace and blackcurrant powders. The final diets were not subjected to any heat processing.

Given these considerations, the approach in pigs prioritized "antibacterial feedstuff" (no extracts) over feed additives due to organic production restrictions and the potential for broader use, as feed additives necessitate stricter regulatory compliance.

Study-III: Palm kernel oil fatty acids and essential oils

The plant materials for this study were selected based on available literature and further applicability. Thus, it was prioritized that plant materials could be sourced from animal nutrition companies. The lauric acid rich MCFA source and essential oils were chosen based on the composition of individual substances with reported antibacterial properties and toxicological safety.

The hydrolyzed lauric oil and essential oil used for the in vivo experiment were analyzed for fatty acid composition and eugenol content, respectively. The oils were kept ~2 C° until use. In the feed mill it were embedded withing soybean oil fraction for improving of mixing in the diet. The feed was cold pelleted to reduce oil volatilization.

In contrast with the approach used with pigs, the plant materials used in the study with chickens would be considered as feed additives and thus should follow the respective EU regulations.

2.3 ANTIBACTERIAL EFFICACY ASSESSMENT

2.3.1 In Vitro Methods for Antibacterial Screening of Plant Materials

Culturing Techniques

Standardized guidelines for antibacterial testing from for example CLSI, BSAC, and EUCAST[12] provide standard methods for antimicrobial testing of conventional drugs. A summary of common methodology is presented in **Table 2.1**. The disc diffusion technique is popular for its simplicity and low cost, involving the placement of an antimicrobial disc on bacteria-inoculated agar to observe clear zones of inhibition (Tan and Lim, 2015). However, it has limitations, such as false negatives for non-polar compounds and inability to determine quantitative metrics like MIC (Tan and Lim, 2015). The broth microdilution method involves serial dilutions in broth and

[12] Clinical and Laboratory Standards Institute (CLSI) [formerly known as National Committee for Clinical Laboratory Science (NCCLS)], British Society for Antimicrobial Chemotherapy (BSAC) and the European Committee for Antimicrobial susceptibility testing (EUCAST)

allows precise MIC and MBC determination but faces challenges with non-polar compounds and heat-labile extracts (Fankam and Kuete, 2023). Agar dilution and multi-channel oxygen meters offer alternative quantitative methods (Fankam and Kuete, 2023). Standardizing inoculum size is crucial for consistency, with common standards being 10^6 CFU/plate for disc diffusion and 5×10^5 CFU/ml for broth microdilution (Fankam and Kuete, 2023). MIC determination varies among guidelines and is critical for resistance surveillance (Fankam and Kuete, 2023). Phenolic compound-induced bacterial aggregation can complicate MBC and time-kill studies.

Overall, each method has its strengths and weaknesses, thus careful optimization for specific applications to ensure accurate antibacterial assessment are warranted. The choice of method depends on factors such as solubility (hydrophobic vs. hydrophilic), the characteristics of the organism, and available resources.

Table 2.1 Summary of some popular in vitro antibacterial screening methods. Some methodologies can be adapted for either macrodilution (macro) or microdilution (micro) methods (Tan and Lim, 2015, Fankam and Kuete, 2023).

Method	Description	Advantages	Limitations	Considerations
Agar disk-diffusion	Antimicrobial test using discs	Simple, low cost, interpretable results	Cannot distinguish bactericidal vs. bacteriostatic not suitable for MIC determination	Not mechanized, labor-intensive, suitable for testing large numbers of bacteria
Agar well diffusion (AWD)	Antimicrobial test using wells	Simple, low cost, adaptable for MIC determination	Time-intensive, less effective for hydrophobic compounds	Suitable for hydrophilic compounds, labor-intensive
Bioautography TLC: Thin Layer Chromatography	Variation of AWD but in TLC plate	Simple, inexpensive, rapid, uses small sample amount	Not suitable for high-capacity screening	Requires appropriate solvents and visualization methods
Agar dilution	Antimicrobial concentration in agar	Simple, fast, suitable for water-soluble/insoluble samples	Labor-intensive, high sample requirement	Good for fastidious organisms, large amounts of test compound needed
Broth dilution	Antimicrobial concentration in broth	Quantitative results (MIC), reproducible, space-efficient (micro)	Tedious sample prep (macro), not suitable for hydrophobic samples (micro)	Microdilution is more economical and automated, precision affected by sample preparation
Agar microdilution	Agar dilution in microplate	Time/cost-effective, suitable for hydrophobic samples	Requires precise sample preparation	Combines advantages of agar and microdilution methods
ATP bioluminescence assay	Measures bacterial ATP production.	Rapid, quantitative.	Difficult comparison with other studies, specific reagents.	Suitable for rapid testing, provides relative light unit measurements
Flow cytometric/fluorescence	Uses dye-staining for viability	Rapid, detailed viability assessment	High equipment cost, limited accessibility	Effective for rapid antimicrobial resistance detection.
Microfluidic methods	Growth in microchannels	High throughput, real-time, low sample consumption	Specialized equipment, complex sample preparation	Ideal for advanced screening, requires microfluidic setup

In Vitro Gastrointestinal Simulation

One drawback of conventional in vitro antibacterial testing is the dissimilarity with in vivo conditions. These methods may underestimate the impact of the gastrointestinal environment on plant materials, potentially misrepresenting their effectiveness (Vermaak et al., 2009). For instance, hydrochloric acid and GIT enzymes can cause chemical modification and inactivation of compounds, reducing their bioavailability (de Carvalho et al., 2021). The interaction of ingested feed with the gut microbiota adds another layer of complexity to this process (Iqbal et al., 2020). Thus, the use of in vitro fermentation systems has been suggested as an alternative to study the gastrointestinal impact of interventions under a setting that more closely resembles that of the GIT. However, in vitro methodologies that simulate GIT condition are less standardized and more specialized than simple antibacterial testing.

In vitro systems could be static/dynamic and single-/multi-step, based on the simulation conditions and the number of different GIT compartments simulated in bioreactors (Sommerfeld and Santos, 2021). Furthermore, the simulated GIT conditions could be based on simulated digesta fluids or samples obtained from euthanized animals. Intestinal fermentation models incorporate fecal (Gresse et al., 2021b) or digesta (Bellerose et al., 2023) inoculums to simulate gut microbiota dynamics.

Efforts for standardization of techniques has been made by adaptation of human models for use in monogastric animals (e.g. baby-SPIME based on SHIME®; Dufourny et al. (2019)). Recent developments include the feature of reproducing the mucus-associated microbiota of piglet colon using mucin beads. For instance, the MPigut-IVM (Mucin associated Piglet Gut In vitro Model) developed by Gresse and collaborators have been optimized for the study of pig postweaning events in vitro including pathogen challenge (Gresse et al., 2021b) and feed deprivations (Gresse et al., 2021a).

Cell-Based Models

Porcine and chicken intestinal epithelial cell models have also been used for the study of plant materials (Marks et al., 2022). For instance, the IPEC-J2 cell line, derived from the jejunum of neonatal piglets, is widely used due to its ability to form a polarized monolayer with tight junctions and microvilli, closely mimicking the porcine intestinal epithelium. The IPEC-J2 models have been used to investigate antioxidative and anti-inflammatory effects plant phytochemicals providing mechanistic insights of activity (Ling et al., 2016, Pomothy et al., 2020). However, IPEC-J2 models does not fully resemble the intestinal environment, lacking for example goblet cells and brush border enzymes (Marks et al., 2022). In chickens, the establishment of cell lines model has been less successful but recent efforts include primary chicken epithelial cell cultures (Marks et al., 2022). Organoid and Organ-on-chip models are also recently being developed for the study of nutritional interventions (Costa et al., 2024) and the study of ETEC-host interactions (Vermeire et al., 2021). Organ-on-chips replicate the GIT environment, enabling detailed studies of nutrient absorption, pathogen interactions, and the effects of interventions (Costa et al., 2024). Organoids, derived from stem cells, mimic the intestinal epithelium's structure and function (Costa et al., 2024). While offering advantages over cell line models, currently these models face challenges in maturation, standardization, and complexity.

Ex vivo methods like the intestinal segment (Ripken and Hendriks, 2015) or Ussing

chambers are also utilized in pigs to measure nutrient absorption and intestinal permeability (Park et al., 2020). However, their adoption has been limited due to technical complexity.

In this thesis

Study-I and 2: In vitro fermentation of stomach content and pathogen inoculum

The study itself did not incorporate in vitro antibacterial determination. However, the background testing incorporated a series of antibacterial screening of several plant materials against different strains of enterotoxigenic *E. coli*. The method used utilized stomach content obtained from pigs and incubated under constant agitation in stomacher bags in which ETEC inoculum and testing materials were added. The plant materials used for the experiment in vivo were analyzed in vitro for antibacterial efficacy against several ETEC strains using this method as well.

Study-III: Broth-agar microdilution with adaptations to use oils

For the in vitro study, a broth microdilution with addition of 5% agar was used for the assessment of antibacterial efficacy of fatty acids and essential oils, following recommendations from: Hood et al. (2003) and Ahmed Khan and van Vuuren (2021). The method allows for determination of MIC which was important for further combination assays and establishment of interactions.

The refining of the method required testing of materials used for sample preparation to ensure oils dissolution, particularly the following considerations:

- The use of broth growing media with agar inclusion to aid on bacterial cells suspension.
- Selection of a solvent for the oils; ethanol, acetone and DMSO solutions were assessed. An aqueous solution of 50% DMSO was chosen due to best solubility both essential oils and fatty acids.
- Selection of a surfactant: tween 80 was added to the growing media, which allowed for oil solutions to mix well with the growing media.

2.3.2 The Study of Interactions

Interactions can be classified as antagonistic, additive/indifference, or synergistic. Additive and indifference indicate lack of interaction, while an antagonism occurs when the interaction of the factors results detrimental for the outcome measure. On the other hand, synergy occurs when the interaction results in potentiation of the expected outcome.

Synergy is a ubiquitous term, fairly simple to describe, but far more complicated to evaluate experimentally. Qualitatively it could be described as *a cooperative action*. Quantitatively as *a supra-additive effect* (Geary, 2013) — colloquially: "*The whole is greater than the sum of its parts*" or "*when 1+1 > 2"*. Complexity arises when applying the concept to plant materials where several active substances may contribute to the expected outcome. Furthermore, when combining multiple plants, the interactions among individual compounds can quickly become intricate (van Vuuren and Viljoen, 2011).

Although considerations exist, the current preferred evaluation tool for assessment of interactions is the isobole method (Caesar and Cech, 2019). The isobole method can be represented graphically using isobologram in a cartesian space where each axis represents the concentration of the combination tested **(Figure 2.2**). The points are constructed by combinations and the result function would indicate the degree of the interaction. The interaction can be classified as indifference (additivity) when action of combination is fractionally equal, while of the action is shown below the indifference line, the combinations act synergistically, and action above the indifference line would indicate antagonistic action. To assess interactions among antimicrobial substances in vitro, this method can be adapted (Leber, 2020). The intersection with each axis then represents the MIC of the individual substances.

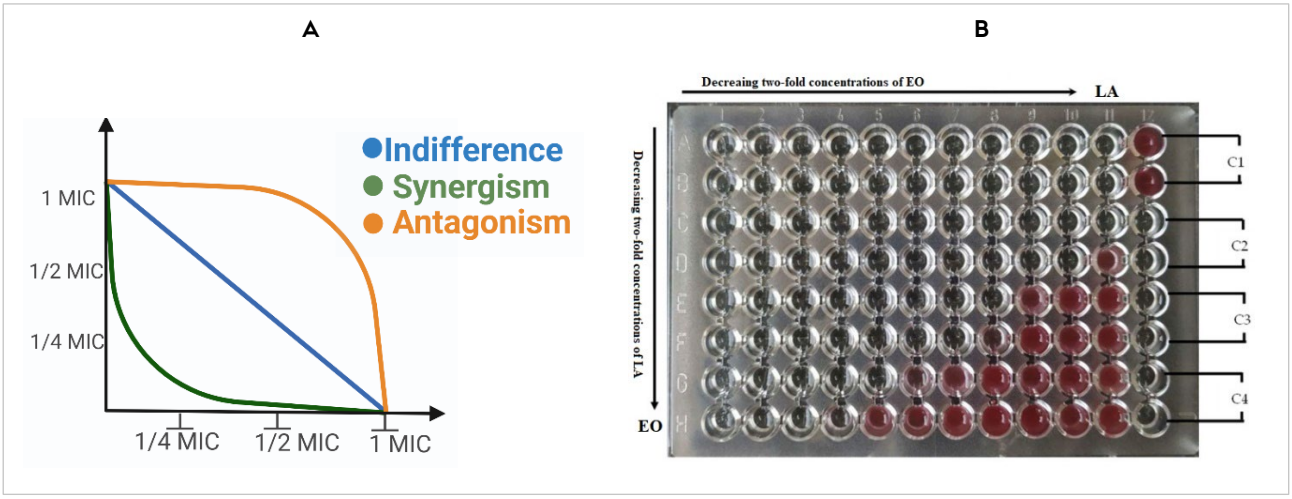


Figure 2.2 Example of isobole method implemented using a checkboard method of microdilutions for assessments of two-way antibacterial interactions. (A) Isobologram for visualization of antagonistic, additive, and synergistic interactions of antimicrobial agents. Each axis represents the concentration of the tested combination, and the intersection with the axis represents the MIC of the individual substances. The interaction can be classified as indifference (additivity) when the effect of the combination is fractionally equal (blue). If the effect is below the indifference line, the combination acts synergistically (green). If the effect is above the indifference line, it indicates antagonism (orange). (B) Illustration of the checkboard method for antibacterial synergy testing microdilutions in a 96-well plate. This example illustrates the combination of two products: "LA" and "EO". Each plate is prepared in such a way to vary LA 1.5-fold concentrations across rows (from A to G; repeated in 1 to 11) and EO along columns (from 1 to 11; repeated in A to G). Column 11 contains only LA, while row H contains only EO. Column 12 contains controls: bacterial growth (C1), EO stock sterility (C2), LA stock sterility (C3), and medium sterility (C4). Modified from: Nikolic et al. (2022) (CC BY 4.0)

The in vitro evaluation of interactions of antimicrobial combinations can be determined using four primary methods: the checkerboard method, multiple-combination bactericidal antimicrobial testing, time to kill curve assays and E-test (Doern, 2014). The checkerboard method evaluates the activities of antimicrobial combinations at relevant concentrations using serial 2-fold dilutions in a 96 well plate (Doern, 2014). Decreasing concentrations of the individual agent are arranged to decrease vertically or horizontally, creating combinations along the gradient (Leber, 2020). The individual MIC should be determined beforehand and be included in the combination assay. The data can be analyzed using the fractional inhibitory concentration index or isobologram (Leber, 2020). This method only tests antimicrobials for a fixed incubation time and cannot assess combinations of more than two antimicrobials simultaneously (Doern, 2014).

Multiple-combination bactericidal testing evaluates combinations of two to four antimicrobials at relevant concentrations using 96-well plates inoculated with the test organism (Doern, 2014). Given the increased number of combinations, only fixed concentrations are assessed. Time to kill assays assess antimicrobial activity over 48 hours, collecting bacterial samples at intervals to determine the rate of killing (Doern, 2014). Synergy is defined as a >2 log10 increase in activity by the combination compared to the active component alone (Doern, 2014). The E-test method uses gradient diffusion to determine MIC. Interactions are determined by intersecting or sequential E-test strips (Doern, 2014). For the latter similar limitations applied for diffusion testing mentioned in the previous section, including difficulties to handle hydrophobic substances.

In this thesis

Study-I: In vitro fermentation of stomach content and pathogen inoculum

The paper did not include in vitro antibacterial determination. However, in earlier work from the MAFFRA-II project, the interactions among the plant materials were assessed by testing specific combinations and concentrations of the plant material individually and in combination. The assessment of interaction was assessed by comparing the ETEC growth/inhibition after 6 hours of growth when using the individual plants or the combinations.

Study-III: Adapted checkboard method

For the in vitro study, the checkerboard method was used following the determination of individual MICs. Combinations of the lauric oil and the two best-performing essential oils were used for the combination assay. Growth assessment was based on growth curves constructed by measuring optical density and microscopic inspection after overnight growth. Isoboles were used to determine interactions.

2.3.3 In Vivo Methods for Antibacterial Screening of Plant Materials

Controlled Challenge Studies

Challenge studies are crucial for assessing the potential efficacy of novel strategies to control PWD or ANE in vivo. Reproducing diseases in the laboratory with symptoms similar to those observed in the field is often challenging (Fairbrother et al., 2005, Prescott et al., 2016a). However, the growing interest in alternatives to antibiotics has led to the refinement of experimental challenge models (Shojadoost et al., 2012, Prescott et al., 2016b, Luise et al., 2019a).

Postweaning Diarrhea Caused by Enterotoxigenic E. coli in Pigs

Several considerations have been made regarding the experimental reproduction of ETEC F4 and F18 challenge models for PWD (Luise et al., 2019a). The molecular testing of specific markers of genetic susceptibility is used for selection of litters susceptible for the pathogenic strain, i.e. genetic markers for specific intestinal receptors (Luise et al., 2019a). In addition to genetic screening, pathogen-specific immunization of piglets and sows should be considered, as maternal immunity can impact piglet response to ETEC challenges. Selecting piglets from non-immunized sows is recommended for infection studies.

Preconditioning procedures such as administration of antibiotics or fasting prior to inoculation are used in some instances to standardize the pig's condition before infection, but they are more common for vaccination studies (Luise et al., 2019a). When these procedures are used the postweaning physiology is altered and therefore may reduce the similarities with natural infection.

The timing of the inoculation is another key factor as bacterial receptor expression in the intestines is shown to be time dependent (Luise et al., 2019a). In the field ETEC infections often coincide with weaning due to stress and reduced passive immunity, leading to intestinal dysbiosis (Luise et al., 2019a). Consequently, in most studies the piglets are inoculated on weaning day or shortly after to exploit this stress period (Luise et al., 2019a, Duarte et al., 2023b). However, other studies prefer inoculation 3 to 7 days after weaning when maternal immunity is reduced and feed intake has increased (Wong et al., 2022). The dose and number of ETEC

inoculations varies widely across studies (Luise et al., 2019a, Dahmer et al., 2023). A single dose of approximately 10^9 CFUs can cause infection, and generally, higher doses have been used in pigs 28 days and older (Luise et al., 2019a, Dahmer et al., 2023). However, although the factors involved in inoculation are recognized as critical, they are usually based on local optimizations by each research group, leading to high variability (Luise et al., 2019a).

Necrotic enteritis Caused by netB+ C. perfringens in Chickens

The experimental reproduction of ANE has also been refined to successfully replicate the main features of the disease with particular relevance of predisposing factors besides the inoculation with a of a pathogenic *netB+ C. perfringens* strains (**Figure 2.3**). Key factors include the choice of infectious agents, such as using *C. perfringens* type G alone or in combination with coccidia and/or infectious bursal disease virus, and the administration route, bacterial load and number of inoculations (Prescott et al., 2016b). Dietary manipulations also play a crucial role in influencing disease severity, finely ground feed, high trypsin inhibitors, mycotoxins and diets high in soluble NSP and animal-origin protein increasing severity of the infection (Shojadoost et al., 2012, Moore, 2024).

Male birds are usually selected for ANE challenge studies as it has been reported an increased susceptibility of male chickens (Shojadoost et al., 2012). Immunization of the parent flock has shown to reduce disease severity; thus, chicks should ideally come from unvaccinated parent flocks (Shojadoost et al., 2012). Furthermore, challenge occurs around 2–3 weeks of age should ensure low maternally-derived antibodies in chicks (Shojadoost et al., 2012).

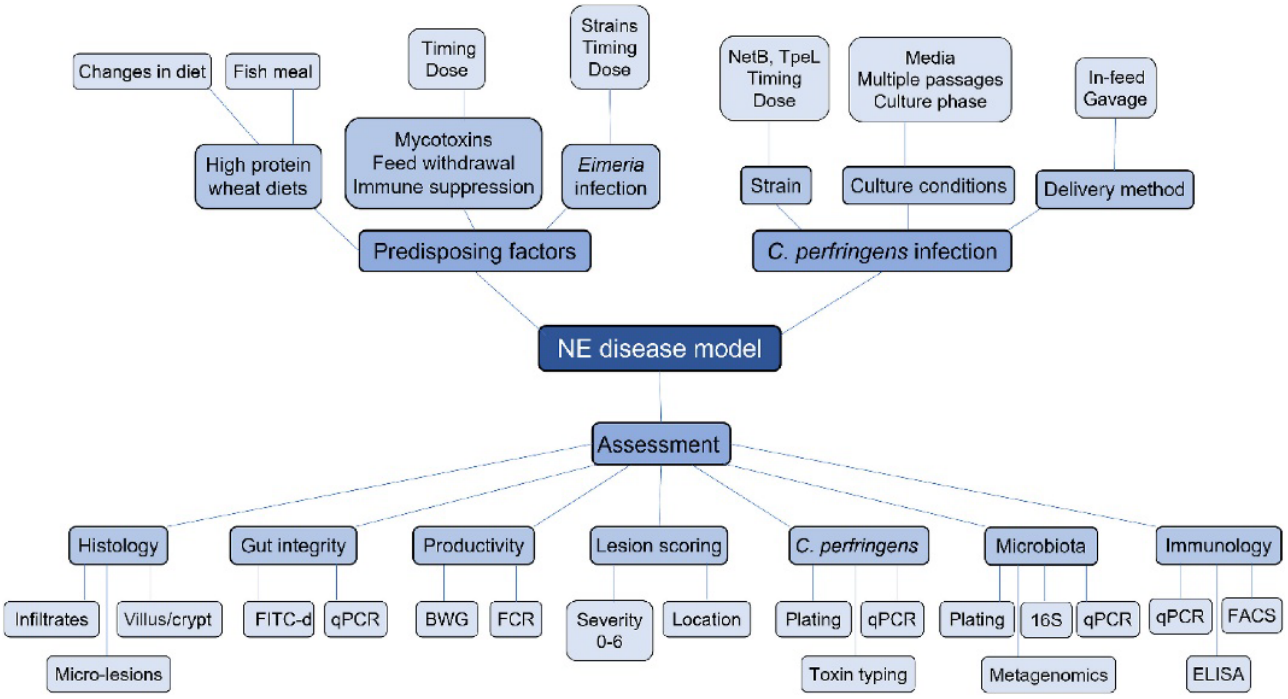


Figure 2.3 Summary of experimental reproduction of necrotic enteritis in broilers and analytical methods used to assess its impact. TpeL = toxin perfringens large; NetB = necrotic enteritis toxin B-like; FITC-d = fluorescein isothiocyanate-dextran; BWG = body weight gain; FCR = feed conversion ratio; FACS = fluorescence active cell sorting. Extracted from: Moore (2024). (CC BY-NC-ND 4.0)

Natural Challenge Studies

Effects on disease prevention or dietary interventions in the context of PWD and ANE have

also been studied using natural infection models. These experiments typically involve conventional housing conditions where the onset of disease is expected due to historical prevalence, low sanitary conditions, and stress events common in conventional management settings (Massacci et al., 2020). Although these experiments more closely resemble commercial settings, the bacterial load and disease onset may not be homogeneous among the animals, potentially complicating the analysis of outcomes.

Acute water and feed deprivation has also been used as a model of postweaning stress, resulting in effects in growth performance, inflammation and diarrhea development, not necessarily related to bacterial infections (Horn et al., 2017). Cohort and growth performance studies in larger industrial settings are often considered as final "proof" of effectiveness as experimental disease models could not be applicable to commercial settings (Moore, 2024).

In this thesis

Study-I and 2: ETEC F18 challenge study

Organic piglets (7 weeks old) were selected for susceptibility to ETEC F18. The pigs did not receive any previous antibiotics or feed restrictions. The challenge used two oral administrations on consecutive days (8ml/day; 10^9 CFU/ml), the dose is higher than that used normally in the research group as pigs were weaned older than conventional pigs.

Study-III: Necrotic enteritis challenge study

Male 1-day-old broiler chickens (Ross 308) were obtained from a commercial hatchery. The disease model involved a wheat-fish meal diet in the grower phase, an overdose of a coccidiosis vaccine, followed by *C. perfringens* infection. Birds were orally inoculated on 18-20 d with NetB+ C. perfringens (strain S48;10^8 CFU/bird/day).

2.4 MEASURING EFFECTS ON GASTROINTESTINAL MICROBIOTA AND ECOSYTEM

2.4.1 Assessment of Gut Pathogens and Microbiota

Traditionally, culture-dependent techniques of selected microorganisms were used to assess the effect of intervention on the gut microbiota, but these are limited as not all microorganisms can grow in vitro. Culture-independent methods can provide a more comprehensive analysis (Chatterjee et al., 2024). These methods include PCR-based DNA profiling, quantitative PCR, fluorescence in situ hybridization, flow cytometry, DNA microarrays, and next-generation sequencing. In nutritional and health studies of monogastric animals (and in general gut microbiome studies), 16S ribosomal RNA (rRNA) has become popular due to the increasingly lower cost of sequencing, important data output, and particular advances in interpretability (Bailey et al., 2019). The 16S rRNA gene contains nine hypervariable regions (V1-V9), with the V3 and V4 regions being commonly targeted in microbial community analysis due to their balance between sequencing diversity and technical manageability (Thumann et al., 2019, Satam et al., 2023).

While 16S rRNA sequencing provides taxonomic information, it does not offer functional insights. Whole metagenomic sequencing addresses this limitation by cataloging all genes through the random sequencing of all DNA extracted from a sample (Thumann et al., 2019). This approach can be used for detailing functional capabilities of the microbial communities and describing metabolic pathways. However, a drawback of whole genome sequencing is the

reduced development on bioinformatics pipelines and the fact that no functional information is available for several genes, creating a "dark matter" phenomenon (Thumann et al., 2019). Furthermore, functional information is only potential as actual gene expression is not measured.

Meta-transcriptomics offers a comprehensive view of gene expression profiles. This approach captures the total mRNA to provide information on the active functional profile of a given microbial community (Thumann et al., 2019). Some limitations of the method are interference with ribosomal RNA, instability of mRNA, and differentiation between host and microbial RNA (Thumann et al., 2019).

Bacterial Quantification Using Quantitative Real Time PCR

The routine method for describing ETEC proliferation during PWD has relied on individual sampling and bacterial culture followed by either serotyping or PCR detection of virulence genes (F4/F18 and ST/LT) in samples collected from intestinal content or feces (Weber et al., 2017). Blood agar is used as hemolytic activity of *E. coli* is a recognized virulence marker, and *E. coli counts* in a "pure" culture are landmark of colibacillosis (Francis, 2002).

Recently, qPCR analysis has emerged as a powerful quantification tool by using specific primers (Weber et al., 2017). Specific primers can be selected for quantification of bacterial groups such as *Lactobacillus*, *E. coli*, *C. perfringens* providing a more efficient and scalable solution than culture-based methods (Takahashi et al., 2008). Furthermore, virulence genes can be also measured providing further insights and confirmation of challenge success (Rhouma et al., 2017c, Dahmer et al., 2023). Similarly, real-time PCR assays targeting the α-toxin-encoding *plc* gene and *netB* genes have also been used as alternative to culture-based methods for quantification of *C. perfringens* in necrotic enteritis experiments (Abildgaard et al., 2010a). Virulence genes can be also measured for confirmation of phenotypic features of pathogenic strains (Abildgaard et al., 2010b).

Genomic counts by qPCR counting have been favored due to scalability, reduced cost, specificity and repeatability (Wang et al., 2017). Another advantage of the technique is that the extracted DNA used for the qPCR assay can also be used for further analysis such as 16S rRNA gene sequencing. A limitation of the technique is the inability to differentiate DNA of viable cells from dead ones. The use of dyes for assessment of membrane integrity in conjunction with qPCR has been suggested for remedy of this limitation (Zi et al., 2018).

Bacterial Microbiota Profiling Using 16S rRNA Gene Sequencing

The 16S rRNA gene sequencing is effective to achieve taxonomic resolution up to the genus level but often falls short at the species level due to homology between some species, which limits taxonomic resolution (Bars-Cortina et al., 2023). Hence, the method can be used to compare the abundance of bacteria at the genus level in different states.

Additionally, gut microbiota research has gained major insights from ecological sciences. It is now widely acknowledged that a diverse microbiome represents a healthy homeostatic condition (a healthy ecosystem), whereas the domination of a few microbial species indicates diseased conditions (a diseased ecosystem) (Chatterjee et al., 2024). The most common ecological measures used in microbiome studies are α- and β-diversity. In the context of gut microbiology, most diversity metrics relate to community richness (the number of different taxa),

evenness (equity in the representation of the taxa within the community), and phylogenetic relationships among species (Tipton et al., 2019). Different indexes take into account one or a combination of these features.

Metrics of α-diversity includes Observed (also called Richness), Shannon, Inverse Simpson, Gini, and Chao, among others (McMurdie and Holmes, 2013). The Observed diversity metric simply counts the number of distinct taxa observed, providing a basic measure of species richness. The Shannon Index takes into account both the number of species and their evenness (McMurdie and Holmes, 2013). The Inverse Simpson Index and the Gini-Simpson Index emphasize evenness, indicating the probability that two randomly selected individuals belong to different taxa, with higher values representing greater diversity (McMurdie and Holmes, 2013, Daly et al., 2018). Chao estimates species richness by considering rare species, providing a more exact picture of diversity in samples where many species are infrequently observed (McMurdie and Holmes, 2013).

On the other hand, β-diversity or compositional dissimilarity is used for comparison of the overall composition of gut microbiota profiles from different samples (Tipton et al., 2019). Richness, evenness, or phylogenetic relationships among species can also be integrated in the measure (Tipton et al., 2019). For example, Bray-Curtis distance considers relative abundances, whereas Unifrac distance accounts for phylogenetic relationships among species as well (Tipton et al., 2019). Temporal variance as a feature of the microbiota can be also assessed and it is termed as volatility (Bastiaanssen et al., 2021). Microbiota volatility can be measure using the degree of dissimilarity of samples in between timepoints (Bastiaanssen et al., 2021). Further analysis includes differential abundance, co-occurrence networks, correlation analyses, supervised and unsupervised learning methods for biomarker discovery, among others (Tudela et al., 2021). The adequacy of the type analyses is constrained by the nature of the data obtained and the experimental desing.

The data obtained from 16s rRNA amplicon sequencing is compositional, thus requiring careful analysis and interpretation (Tudela et al., 2021). For example, data is often normalized which introduces dependency, skewing results when abundant species disappear (Tudela et al., 2021). Another limitation of this method is given by the variable number of 16S rRNA gene copies in members of the phyla Firmicutes and Bacteroidetes, which results in an over-representation of such groups (Tudela et al., 2021). Finally, an important consideration is the susceptibility of bias introduction on any of the steps of the whole workflow from the sample collection to the bioinformatic processing (**Table 2.2**), thus making it particularly relevant to follow best practices and standardized procedures (Combrink et al., 2023).

Table 2.2 Important considerations in 16s rRNA gene sequencing studies. Based on (Combrink et al., 2023).

Item	Considerations	In this thesis
Sample Collection and Preservation Techniques	- Use sterile techniques to avoid contamination. - Preserve samples to prevent microbial growth. - Preservation methods: cold storage, buffer solutions, dry storage.	-Fecal samples: Cold until subsampling -Digesta samples: Snap-frozen in liquid N -80 °C storage.

Item	Considerations	In this thesis
DNA Extraction	- Extract high-quality DNA. - Extraction methods: enzymatic vs. mechanical lysis.	NucleoSpin DNA Stool Kit: Lysis by mechanical disruption with ceramic beads
Controls and Mock samples	- Assess technical variation and biases. - Contain known ratios of cells, genomes, or amplicons.	Zymo mock community (DNA) and blank samples incorporated into workflow.
Batch Effects	- Minimize unwanted technical variation (batch effects).	DNA extraction: 96 /batch. DNA sequencing in one batch/study.
Region Selection	- Select the right hypervariable regions (V1-V9) for 16S rRNA gene studies. - Different regions vary in their ability to differentiate taxa.	V3-V4
Library Preparation	- PCR amplifies DNA for sequencing but can introduce errors. - Minimize errors: use high-fidelity DNA polymerases, reduce PCR cycles, manage DNA concentrations.	Done at sequencing company. Study 1 and 2: Novogene Study 3: BGI
Sequencing Technology	- Next-generation sequencing platforms vary in read length, cost, and quality. - Common platforms: Illumina HiSeq and MiSeq, PacBio, Oxford Nanopore.	Study 1 and 2: Illumina HiSeq Study 3: BGI-SEQ DNB
Bioinformatics	- Quality trimming, read assembly, chimera removal, taxonomic assignment. - Tools: QIIME, DADA2, mothur streamline these processes.	DADA2
Phylogenetics	- Provides insights into microbial community evolution and ecology. - Tree construction methods: distance-based, maximum likelihood, Bayesian.	Fast tree algorithm

Study-I: q-PCR bacterial enumerations and 16s rRNA amplicon sequencing

In this study we quantify ETEC bacterial culture and q-PCR on virulence genes. The ETEC counts in feces were quantified using blood agar accompanied by serotyping and targeted qPCR using primers of virulence genes F18 and STb. Furthermore, 16s rRNA amplicon sequencing was used for microbiota profiling of fecal samples.

Study-II: q-PCR bacterial enumerations and 16s rRNA amplicon sequencing

In this study used q-PCR for quantification of bacterial groups using specific primers for: total bacteria, *lactobacillus*, *E. coli* and F18 virulence gene in samples from GIT contents in different sections. Furthermore, samples were used in16s rRNA amplicon sequencing for microbiota profiling.

Study-III: q-PCR bacterial enumerations and 16s rRNA amplicon sequencing

In this study used q-PCR for quantification of bacterial groups using specific primers for: lactobacillus, *E. coli* and C. perfringens, NetB toxin gene in GIT content. Furthermore, samples were used in16s rRNA amplicon sequencing for microbiota profiling.

2.4.2 Measuring Metabolites

Several metabolic pathways are influenced by the gut microbiota which in turn are linked to dietary factors (Valdes et al., 2018). Therefore, the use of plant materials in the diet may impact microbial metabolites and associated pathways in host physiology (**Figure 2.4**). For instance, some of these metabolites could be (1) plant metabolites transformed by gut microbes, (2) microbial transformation of host-excreted compounds like bile acids, and (3) compounds synthesized de novo by gut microbiota, such as SCFAs and vitamins (Thumann et al., 2019).

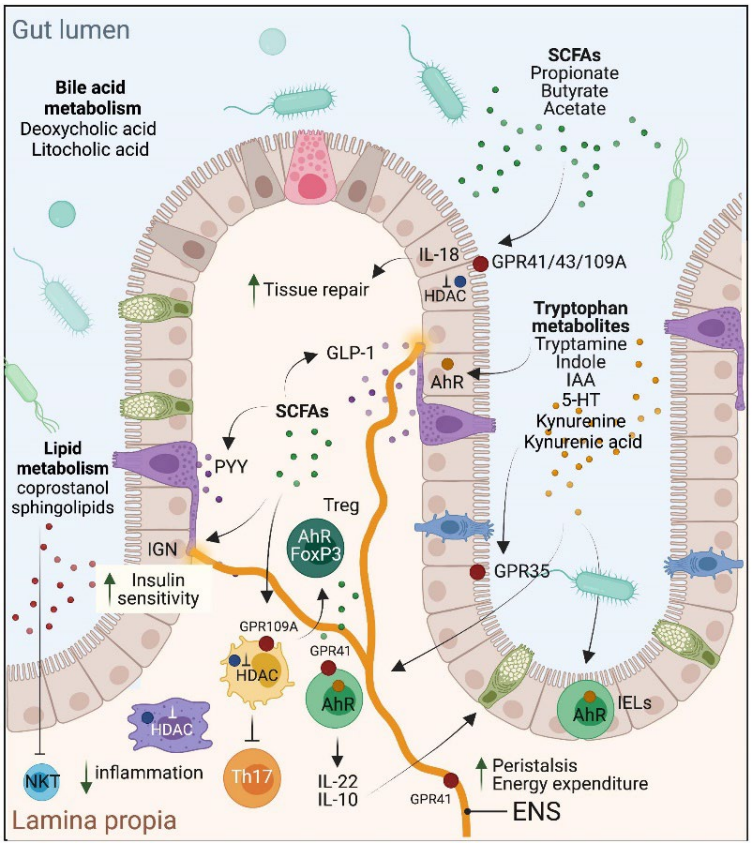


Figure 2.4 Summary of select microbial metabolites on relation with intestinal homeostasis, mucosal immune regulation, and metabolic health of the host. ENS: enteric nervous system. Extracted from: Tudela et al., 2021, (CC BY 4.0).

Short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate are produced during bacterial fermentation of carbohydrates and proteins, while branched-chain fatty acids (BCFAs) like isobutyrate, isovalerate, and 2-methylbutyrate arise from branched-chain amino acids (Gilbert et al., 2018). Undigested monosaccharides are converted to pyruvate, which is then metabolized into acetyl-CoA, succinate, or lactate. Acetate is derived from acetyl-CoA, butyrate from acetoacetyl-CoA, and propionate from lactate or succinate pathways. Butyrate serves as a key energy source for colonocytes, while propionate and acetate are metabolized in the liver (Gilbert et al., 2018).

Ammonia, produced by deamination of amino acids or urea hydrolysis, can disrupt gut health by increasing epithelial cell proliferation and permeability when present in high concentrations (Gilbert et al., 2018). Phenolic compounds, such as phenol and p-cresol, and indolic compounds, like indole and skatole, are produced from aromatic amino acids (Gilbert et al., 2018). Increased levels of these compounds are associated with DNA damage and inflammation (Gilbert et al., 2018). However, recent studies have re-evaluated the roles of these

microbial metabolites, suggesting further roles in host health when not in excess. For example, the conversion of p-cresol into p-cresol-glucuronide by host enzymes has been linked to improved blood-brain barrier integrity (Stachulski et al., 2023). Microbial metabolism of dietary tryptophan is crucial for regulating intestinal homeostasis through AhR (aryl hydrocarbon receptor) activation (Tudela et al., 2021). AhR is activated by tryptophan-derived ligands like indole and indole acetic acid, influencing barrier integrity, immune responses, and peristalsis (Tudela et al., 2021).

Polyamines such as putrescine, spermidine, and spermine, produced by gut bacteria, are important for mucosal development (Gilbert et al., 2018). However, extreme concentrations of these polyamines may affect butyrate uptake and gut health (Gilbert et al., 2018). Hydrogen sulfide (H_2S), produced by sulfate-reducing bacteria from dietary sulfur (Gilbert et al., 2018). Elevated H_2S production can impair mitochondrial function and break down the mucus barrier, potentially contributing to gut inflammation. However, the effects of H_2S on cell proliferation and cytokine production are complex and context-dependent (Gilbert et al., 2018).

Other important metabolites include nitric oxide, lipid metabolites, and bile acids. Nitric oxide, produced from arginine, has both beneficial and harmful effects on gut health, influencing motility, mucus production, and permeability (Gilbert et al., 2018). Bile acids aid in lipid absorption and have systemic effects, including the regulation of metabolism and immune responses through FXR and TGR-5 receptors. Microbial metabolism transforms primary bile acids into secondary bile acids, impacting gut health (Tudela et al., 2021).

Dietary phytochemicals such as polyphenols and organosulfur may be readily absorbed or transformed by microbial modifications (Lawson and Hunsaker, 2018). Therefore, both original phytochemical and degradation products can be found in different metabolomes. Furthermore, some plant-derived metabolites may be treated as xenobiotics by the host, leading the liver to reintroduce phytochemical derivatives to the gut lumen through enterohepatic circulation (Forgie et al., 2019). They can be measured to determine whether their impact on the host health is direct or mediated by the microbiota (Forgie et al., 2019).

Metabolomic Approaches

Metabolites can be measured in multiple specimens, for example in blood, urine, saliva, digesta, or feces, using spectroscopic or spectrometric techniques (Thumann et al., 2019). Metabolomic studies can be targeted analysis of specific compounds or non-targeted approaches to obtain an overview of metabolic profiles (Thumann et al., 2019).

Targeted approaches involved the development of target-specific protocols for extraction and exact measurement. The combination of chromatography and mass spectrometry is used for accurate quantification. Validation in targeted metabolomics methods is important, involving assessments of precision, accuracy, linearity, sensitivity, and specificity (Curtasu and Nørskov, 2023). In contrast with the targeted approach when performing untargeted metabolomics, the aim is to identify several metabolites that are partially or completely unknown within the sample specimen (Tebani and Bekri, 2019, Moco, 2022). The metabolites may undergo further host metabolism, resulting in microbial-host co-metabolites that can be detected through metabolic profiling (Tebani and Bekri, 2019, Moco, 2022). Liquid chromatography-mass spectrometry (LC-MS) or Nuclear Magnetic Resonance (NMR) spectroscopy in metabolomics methods used in untargeted metabolomic studies (Tebani and Bekri, 2019, Moco, 2022).

In this thesis

Study-I:

No metabolites were measured in this study in host samples; however, a targeted method was developed for the measurement allicin in the garlic used for the experiment to ensure antimicrobial activity.

Study-II:

Targeted methods were used for assessment of microbial metabolites in digesta samples including SCFA, indoles, and biogenic amines.

2.5 MEASURING HOST RESPONSES

Host-related indicators are measured in relation to clinical signs of disease and performance parameters, including growth performance, health status, and mortality. The evaluation of growth performance parameters such as average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F) is common in nutrition research. However, the relevance of growth performance as a response criterion in challenge studies depends on the type of study. When a nutritional strategy is used to combat infection growth performance is a key indicator, while those focused on vaccine strategies may concentrate on immunological responses (Dahmer et al., 2023). Additionally, changes in growth performance or mortality often require high statistical power that may not be achievable in disease reproduction experiments (Gebhardt et al., 2020, Dahmer et al., 2023).

2.5.1 Pig Response During Postweaning Diarrhea

The frequency and severity of diarrhea are measured as key response parameters for evaluating ETEC infection. These indicators can be assessed using various methods, such as fecal consistency scoring, fecal DM, and frequency of diarrhea (Luise et al., 2019a, Dahmer et al., 2023). Fecal scoring is the most common measure for assessing diarrhea but has limitations due to its qualitative nature, including scorer bias, threshold definitions, and requiring categorical data analysis (Luise et al., 2019a, Dahmer et al., 2023). Hence, it has been recommended to report more than one assessment of diarrhea with clear threshold definitions (Luise et al., 2019a, Dahmer et al., 2023). Fecal DM is often preferred due to its quantitative nature (Pedersen et al., 2011).

Infection with pathogenic bacteria increases fecal shedding of these organisms, thus ETEC shedding is a widely recognized indicator for evaluating response to infection and effect of interventions (Luise et al., 2019a). Fecal bacterial shedding is evaluated in various ways, including total bacterial shedding, total *E. coli* shedding, ETEC specific shedding, and hemolytic coliforms (Luise et al., 2019a). Specific F4 or F18 ETEC enumeration is the most reliable indicator of ETEC infection (Luise et al., 2019a). Differences in methodologies, such as bacterial culture and quantitative PCR impact sensitivity and reproducibility of results (Dahmer et al., 2023).

Rectal temperature is another clinical indicator for pig health status, which typically ranges from 39.0–39.5°C pre-challenge to > 40.0 °C post-inoculation, then gradually decreasing (Luise et al., 2019a). However, obtaining rectal temperatures may be time-consuming, stressful for the animals, and inaccurate due to watery feces and animal movements (Luise et al., 2019a).

The infection and related intestinal damage trigger local and systemic inflammatory

responses. Indicators of these responses include white blood cell counts, immunoglobulins, cytokines, and acute phase proteins (Luise et al., 2019a, Duarte et al., 2023b, Dahmer et al., 2023). Systemic inflammation is assessed via blood serum or plasma analyses, depending on the severity of the challenge. Acute phase response parameters such as haptoglobin, C-reactive proteins (**CRP**), and pig major acute phase protein **(Pig-MAP**) provide a general assessment of systemic inflammation (Heegaard et al., 2011). Saliva measurements are gaining popularity, but the methods are not yet widespread (Saco et al., 2023).

Pro-inflammatory cytokines like TNF-α and IL-6, measured via ELISA, are particularly responsive during ETEC infection, both in serum and mucosal samples (Dahmer et al., 2023, Duarte et al., 2023b). IgA quantification in blood serum, saliva, and mucosal samples also shows significant responses to ETEC challenges and treatments (Luise et al., 2019b). Other immunoglobulins, including IgG and IgM, have shown varying responses (Luise et al., 2019b).

The occurrence and lasting of PWD indicators vary from study to study (Duarte et al., 2023b, Dahmer et al., 2023). Therefore, measurements are usually recorded from the start of study (at weaning or before inoculation) and last for a least a 3-week period span until visible recovery. The measurements are more frequent during the first week post inoculation and gradually decrease if signs of recovery are obvious.

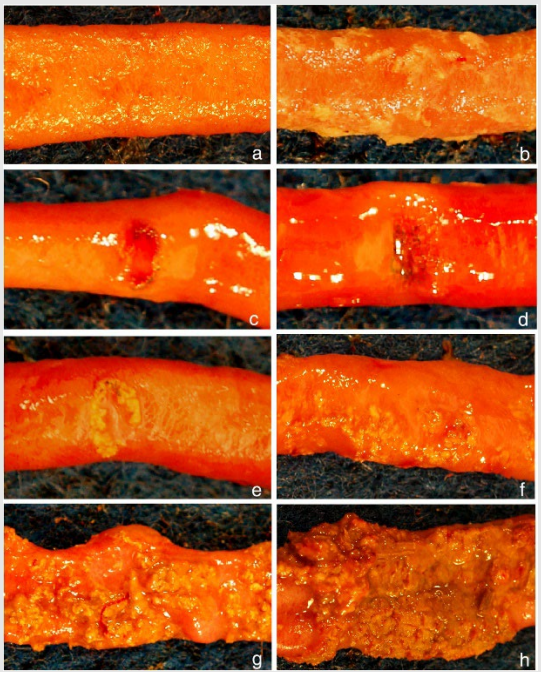
Intestinal morphology is also affected by ETEC infection leading to villi atrophy and increased intestinal permeability (Duarte and Kim, 2022). Histological examination of villus height , crypt depth, and VH:CD ratio provides insights into the impacts of ETEC on intestinal health and the effect of interventions (Duarte et al., 2023b, Dahmer et al., 2023) Additionally, assessing tight junction proteins like occludin (**OCLN**), zonula occludens protein-1 (**ZO-1**), and claudin-1 (**CLDN-1**) can indicate changes in epithelial barrier integrity (Duarte et al., 2023b, Dahmer et al., 2023). Tight junction proteins can be assessed directly by measuring their protein levels using Western blot techniques or indirectly by quantifying their mRNA expression through gene expression assays. Proteomic approaches offer a more comprehensive evaluation by potentially identifying and quantifying multiple proteins simultaneously, including tight junctions (Liu et al., 2016). While proteomic technologies are becoming more accessible and continuously improving, they still face challenges in terms of cost and complexity compared to the widely used Western blotting technique (Hein et al., 2013, Liu et al., 2016, Zampiga et al., 2018).

2.5.2 Chicken Response During Necrotic Enteritis

Intestinal lesion scoring is the primary measure for assessing the progression of ANE. The lesions may be localized along the small intestine, primarily appearing in the jejunum (Prescott et al., 2016b). Shojadoost et al. (2012) proposed a scoring system with a severity index ranging from 0 to 6 assessing the intestinal damage caused by ANE (**Figure 2.5**). A score of 0 indicates no gross lesions, while a score of 1 involves thin or friable walls or a superficial, removable fibrin layer. Scores 2 to 4 represent increasing numbers of focal necrosis or ulceration with non-removable fibrin deposits, ranging from 1 to over 16 foci. Score 5 is characterized by patches of necrosis measuring 2 to 3 cm, and score 6 denotes diffuse, extensive necrosis typical of severe field cases.

Scoring is preferably performed by an experienced pathologist using a blinded system (Prescott et al., 2016b). Histopathological confirmation of lesions can be additionally performed but should not replace scoring, as histologic sections may not represent the entire intestine

(Prescott et al., 2016b). Histological examinations may involve examining infiltrates, villus/crypt structures, and micro-lesions (Moore, 2024). Other indicators of intestinal integrity include FITC-d permeability tests and tight junction protein assays (Prescott et al., 2016b, Moore, 2024). Quantification of *C. perfringens* in fecal droppings or gut contents is also used to assess infection progression (Alnassan et al., 2014). The bacterial load determination can be performed via plating or qPCR (Moore, 2024). The *netB*+ phenotype is critical to disease development thus important for measuring pathological challenge success (Abildgaard et al., 2010b). When *Eimeria* challenge is part of the disease model, oocyst excretion is assessed in fecal droppings (Alnassan et al., 2014).



Score	Observation	Number of lesions
0	No gross lesions	-
1	Thin or friable walls, or diffuse superficial but removable fibrin	-
2	Focal necrosis or ulceration, or non-removable fibrin deposit	1 to 5 foci
3	Focal necrosis or ulceration, or non-removable fibrin deposit	6 to 15 foci
4	Focal necrosis or ulceration, or non-removable fibrin deposit	16 or more foci
5	Patches of necrosis 2 to 3 cm long	Variable
6	Diffuse necrosis typical of field cases	Extensive

Figure 2.5 Intestinal lesions caused by necrotic enteritis in chickens. The pictures of everted jejunal segments illustrating the necrotic enteritis scoring system. a: Score 0, everted jejunal segment with no gross lesions. b: Score 1: No obvious ulcers in the mucosa, but the mucosa covered with a layer of loosely adherent fibrin. c: Score 2–4, excavated ulcer of the mucosa with acute, bright red hemorrhage within the ulcer bed and scant crusting of fibrin around the periphery. d: Score 2–4, excavated ulcer of the mucosa with dark green-black pigment within the ulcer bed and scant crusting of fibrin over the surface. e-f: Score 2–4: excavated ulcers of the mucosa, the periphery of which are covered by thick, tightly-adherent layers of fibrin, necrotic tissue, and inflammatory cells. g-h: Score 5–6, mucosa are covered by large, confluent plaques of fibrin, necrotic tissue, and inflammatory cells (g) to the point where they extend over broad regions of the intestinal mucosa (h). Adapted from: (Shojadoost et al., 2012). (CC BY 2.0).

The primary immune organs in chickens are the thymus, bursa of Fabricius, and bone marrow (Wlaźlak et al., 2023). Secondary lymphoid tissues manage immune responses by activating effector cells. Unlike mammals, chickens lack encapsulated lymph nodes and instead have "diffuse" lymphoid tissue and clusters in structures like Peyer's patches, cecal tonsils, and Meckel's diverticulum (Wlaźlak et al., 2023). Immunological assessments may be performed via gene expression, flow cytometry, and ELISA to measure acute phase proteins, cytokines, and immunoglobulin levels (Moore, 2024). Antigen-specific immunoglobulin concentrations may also be of interest (Dalgaard et al., 2022). Ex vivo immunological analysis is also performed when immunological modulation is of interest (Dalgaard et al., 2022). Common assays include assessing phagocytosis, oxidative burst generation, monocytes and heterophils microbicidal killing and chemotaxis, cell surface molecule expression, and immune-cell cytokine production or mRNA transcription (Dalgaard et al., 2022).

2.6 ETHICAL CONSIDERATIONS

The experimental reproduction of pathogenic infection, such as ETEC-induced PWD and ANE in chickens, causes significant stress and suffering to the animals used. Consequently, bioethical considerations are vital during all phases of experimentation, even more so than in other types of animal experiments. The design of these experiments prioritizes the refinement, reduction, and replacement principles (Graham and Prescott, 2015). Furthermore, the studies require approval under animal care legislation and regulations.

Ensuring the welfare of the animals involves strict adherence to ethical guidelines, including minimizing pain and distress and providing proper veterinary care (Smith et al., 2007). Proper housing, handling, and monitoring of the animals are essential to ensure humane treatment throughout the study (Smith et al., 2007). Furthermore, to make studies transparent and reproducible, investigators should report in accordance with the ARRIVE guidelines (Percie du Sert et al., 2020).

Chapter 3: STUDY OVERVIEW

3.1 STUDY-I
ANTIBACTERIAL PLANT COMBINATIONS PREVENT POSTWEANING DIARRHEA IN ORGANICALLY RAISED PIGLETS CHALLENGED WITH ENTEROTOXIGENIC *Escherichia coli* F18

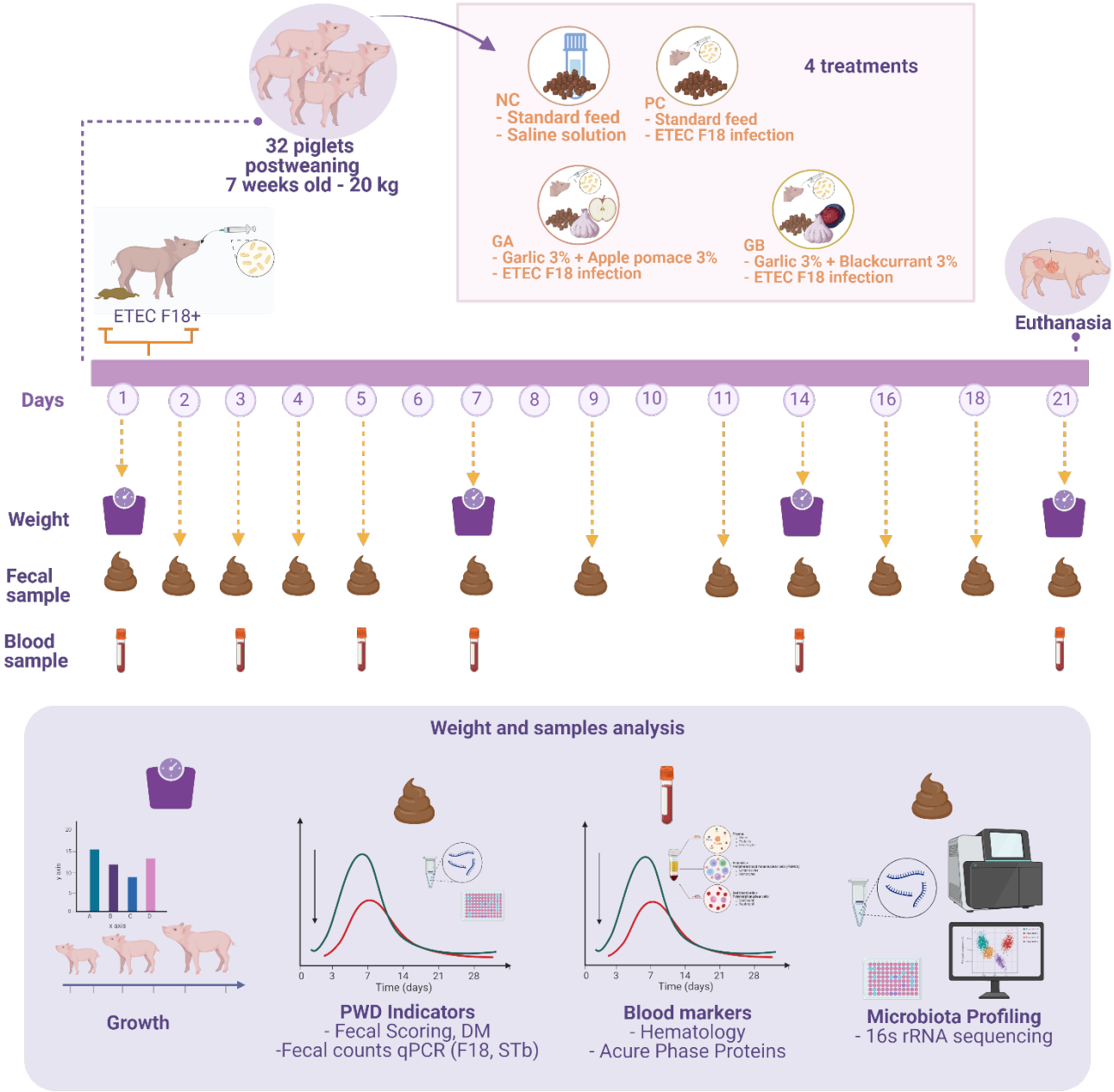


Figure 3.1 Overview of study ***Antibacterial plant combinations prevent postweaning diarrhea in organically raised piglets challenged with enterotoxigenic Escherichia coli F18*** This study investigated the effect of dietary supplementation with garlic in combination with either apple pomace or blackcurrant on growth performance, PWD indicators, blood parameters, and the fecal microbiota profile of organic-raised piglets challenged with ETEC-F18.
Created in BioRender. Jerez, K. (2024) BioRender.com/o35k429

Aim and Hypothesis

We hypothesized that supplementing piglets with garlic and apple pomace, or garlic and blackcurrant, alleviates clinical signs of PWD by selectively inhibiting ETEC and promoting a healthy gut microbiota. The aim of **Study-I** was to investigate the efficacy of dietary supplementation of dried garlic in combination with apple pomace (**GA**) and black currant (**GB**) on the prevention of PWD in organic weaners challenged with ETEC F18.

Key findings

Piglets receiving GA or GB supplements had reduced incidence of diarrhea, lower ETEC F18 shedding, had higher fecal dry matter, and better growth performance compared to the challenged control group (**PC**). The PC group also showed signs of dehydration (increased hematocrit) and systemic inflammation (increased PigMAP), which was prevented in the GA and GB. Furthermore, the fecal microbiota of PC piglets had reduced diversity and increased volatility compared to the supplemented groups. Specific bacteria associated with possible benefits (*Catenibacterium*, *Dialister*, *Mitsoukella*) was favored by the plant's supplementation, while potentially harmful bacteria (*Escherichia*, *Campylobacter*, *Erysipelothrix*) were more abundant in the PC group.

Assessment of the methodology

In this study, we successfully induced PWD symptoms in challenged pigs and observed a clear distinction between unchallenged and challenged control pigs in key parameters like fecal scores, fecal dry matter, ETEC shedding, and growth performance. Overall, this provides sufficient evidence to conclude the efficacy of the plant combinations in preventing disease and displaying potent antibacterial effects against ETEC F-18. Complementary parameters, particularly PigMAP and hematocrit, further validated these endpoint observations. The examination of fecal microbiota over time, along with targeted measurements, provided insights into the antibacterial activity of the supplements and their broader effects on the gut microbial ecosystem.

A second block of this experiment was intended to increase statistical power, but the response to the challenge could not be confirmed in the second group of pigs, so only the first block was analyzed. This, however, did not hinder our ability to draw conclusions, as the response to the challenge and the effect of the treatments were strongly contrasting.

One aspect that could have provided additional valuable information would have been to euthanize and sample some animals on the days of acute response to the challenge. However, as these pigs were "non-conventional" and limited information exists on the use of organic weaners in challenge studies, we could not determine the optimal timepoint for sampling. On the other hand, this led to the decision to obtain more time-point measurements, which enriched the results. Finally, although we assessed allicin content in the garlic powder, measured the pH of the apple pomace, blackcurrant, and diets, and estimated ABC-4 values, the results could have been supplemented by measuring the concentration of organic acids (in powders and diets) and allicin in the diets.

3.2 STUDY-II
EFFECTS OF GARLIC WITH APPLE POMACE OR BLACKCURRANT SUPPLEMENTATION ON THE GASTROINTESTINAL MICROBIAL ECOSYSTEM IN ORGANIC PIGS AFTER WEANING

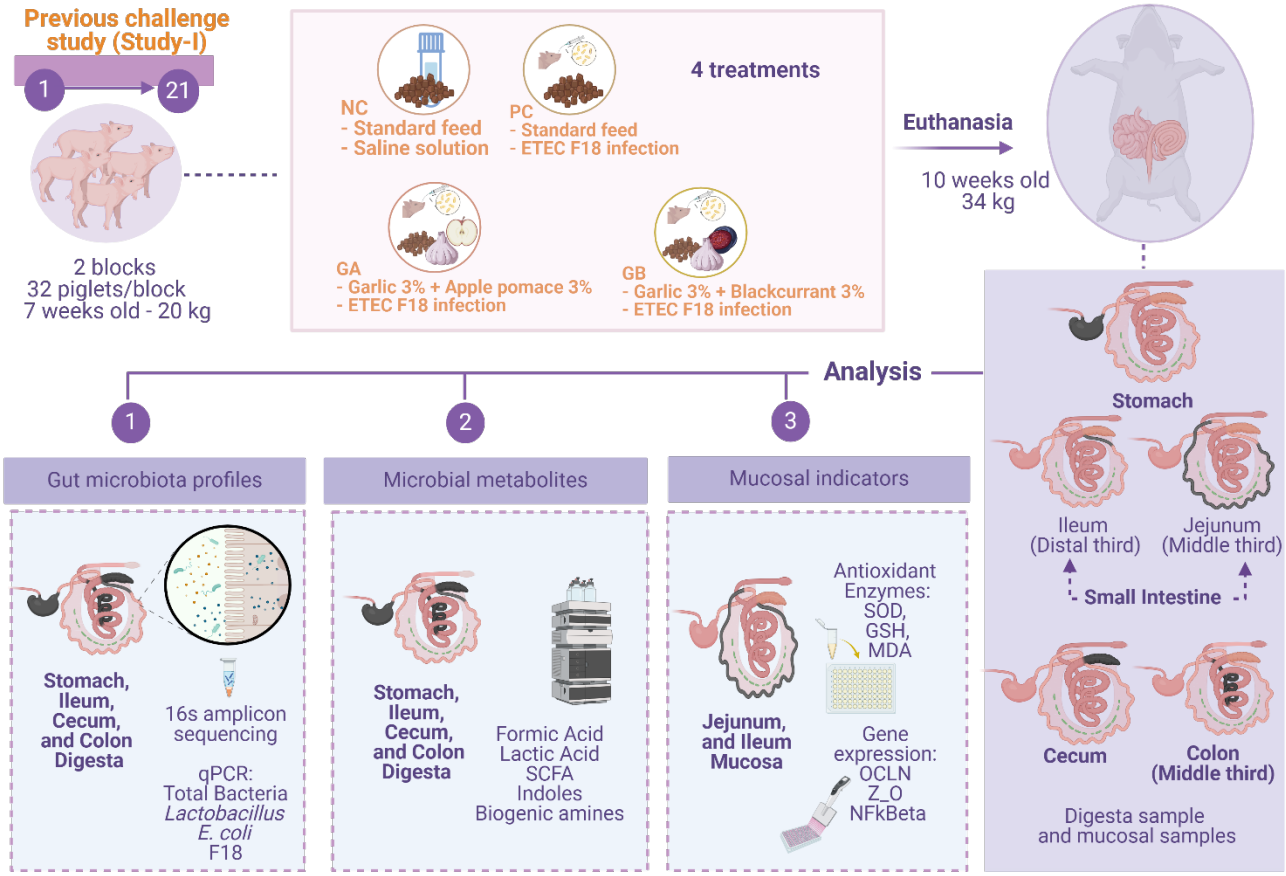


Figure 3.2 Overview of study *Effects of Garlic with Apple Pomace or Blackcurrant Supplementation on the Gastrointestinal Microbial Ecosystem in Organic Pigs After Weaning.* This study investigated the impact of a three-week dietary supplementation of garlic in combination with either apple pomace or blackcurrant on the gastrointestinal microbiota, microbial metabolites, and antioxidant capacity of organic pigs after weaning. Created in BioRender. Jerez, K. (2024) BioRender.com/u79r014

Aim and Hypothesis

We hypothesized that the three-week dietary intake of the plant materials would beneficially influence the GIT ecosystem by selectively promoting the growth of beneficial bacteria and thereby modulating fermentation processes that can prevent detrimental processes like oxidative stress and intestinal barrier disruption. The aim of Study-II of this study was to investigate the impact of dietary supplementation with garlic, in combination with either apple pomace or blackcurrant, on the gastrointestinal microbiota, microbial metabolites, and antioxidant capacity and barrier function of the small intestinal mucosa in organic pigs three weeks after an ETEC challenge at weaning.

Key findings

Overall bacterial numbers were not affected by the treatments, but the microbiota profiles and metabolites were influenced. Weaning exposure to ETEC (**PC**) resulted in increased abundance of potentially pathogenic bacteria at expense of commensal bacteria. Whereas the

dietary supplementation with garlic combined with either apple pomace or blackcurrant in weaned pigs after an ETEC challenge resulted in increased abundance of beneficial bacteria. The GB treatment resulted in greater impacts on specific taxa, resulting in shifted fermentation towards the distal gut (cecum and colon) and favored carbohydrate fermentation over protein fermentation. The plant supplementation increased the activity of antioxidant enzymes (SOD and GSH-Px) in the small intestinal mucosa.

Assessment of the methodology

The method of this study aimed at investigating the extended impact of plant supplementation on the gut microbial ecosystem. The methods chosen allowed for comprehensive assessment of the gut microbial ecosystem, by assessing bacterial numbers (qPCR), the microbiota profiles (16s rRNA sequencing) and targeted microbial metabolites (targeted metabolomics). The inclusion of an unchallenged control (**NC**) allowed for the isolation of the effects of the weaning ETEC challenge, three weeks after challenge.

Additional investigations into the role of plant phytochemicals along the gastrointestinal tract would have provided valuable insights. For example, employing other "omics" approaches could shed light on the mechanistic aspects of interactions between the gut microbiota, plant phytochemicals, and the host. Non-targeted metabolomic approaches could reveal interactions between antimicrobial plant components and gut microbiota. Furthermore, metagenomic approaches could provide insights into the functional aspects of the gut microbiota, beyond mere taxonomic modulation, as different taxa may provide similar functions to the ecosystem, thus increasing taxonomic resolution.

3.3 STUDY-III
EFFICACY OF MEDIUM CHAIN FATTY ACIDS AND ESSENTIAL OILS AGAINST NECROTIC ENTERITIS IN BROILERS

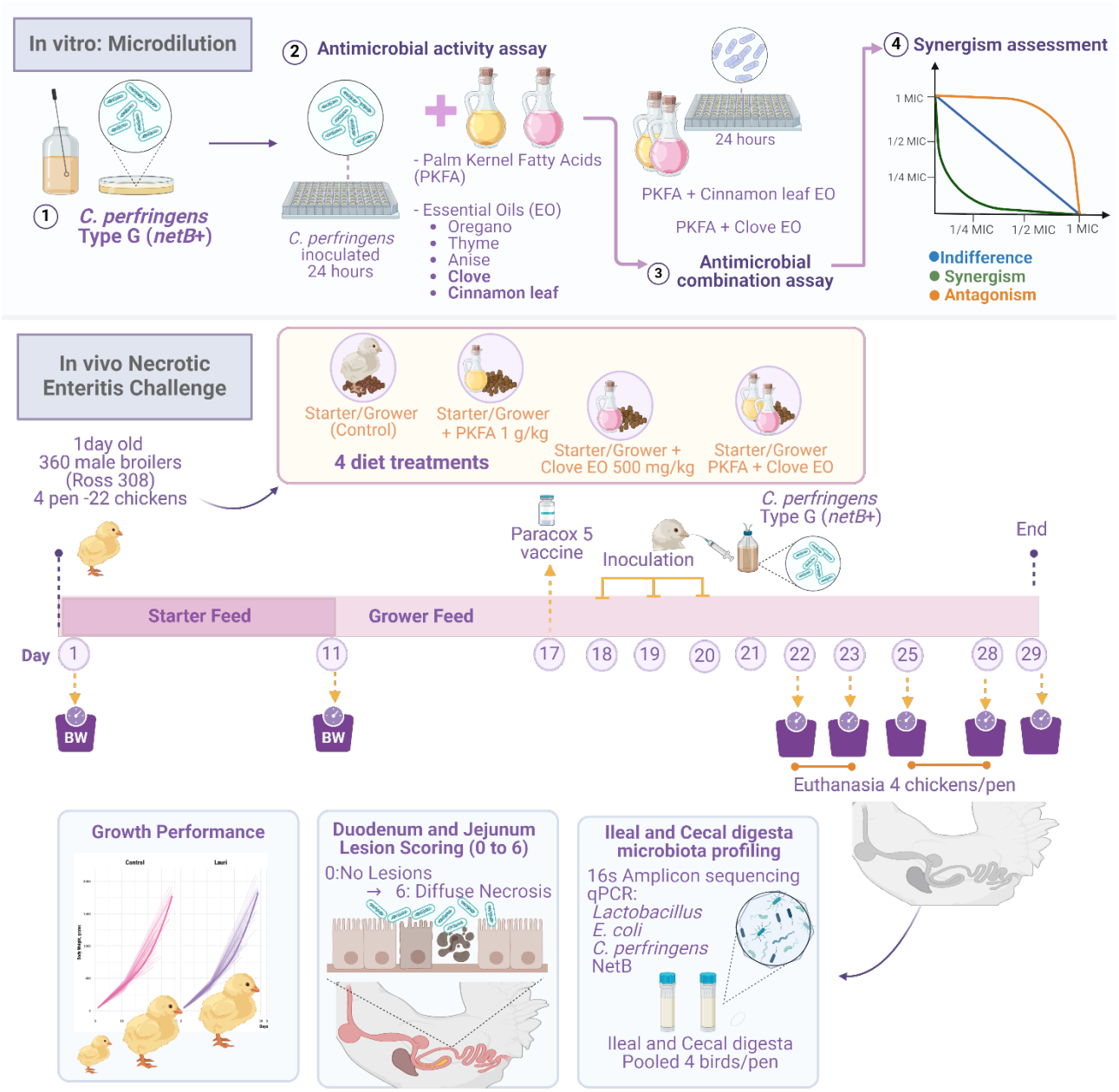


Figure 3.3 Overview of study *Efficacy of Medium Chain Fatty Acids and Essential Oils Against Necrotic Enteritis in Broilers*. This study investigated the combined effects of palm kernel fatty acids as a natural source of MCFAs and essential oils on the course of necrotic enteritis disease and gut microbiota composition in chickens under a necrotic enteritis challenge. Created in BioRender. Jerez, K. (2024) BioRender.com/j03m411.

Aim and Hypothesis

We hypothesize that the combined antibacterial activity of palm kernel fatty acids (**PKFA**) as natural source of MCFAs rich in lauric acid, and essential oils will result in the display of synergistic antibacterial effects against *netB+ C. perfringens* resulting in reduced pathogen proliferation thereby reducing necrotic enteritis disease symptoms and favoring the establishment of a healthy gut microbiota in broilers chickens under a necrotic enteritis challenge. The aim of the

current study is to investigate the combined effects of MCFAs and essential oils on MICs in vitro against *C. perfringens* and in vivo, on intestinal and cecal levels of *C. perfringens*, disease progression and gut microbiota composition in chickens under a necrotic enteritis challenge.

Key findings

The combination of PKFA and clove essential oil showed synergistic antibacterial activity against *netB*+ *C. perfringens* in vitro. In broilers challenged with necrotic enteritis, the combination of PKFA and CEO consistently reduced intestinal lesions caused by necrotic enteritis throughout the study, reduced intestinal *netB*+ *C. perfringens* levels and improved growth performance challenged control. The combination treatment may have led to a faster succession of the gut microbiota and reduced the relative abundance of *C. perfringens* and E. coli in the cecum and ileum at specific time points.

Assessment of the methodology

The study incorporated both in vitro and in vivo experiments to assess the antimicrobial properties of MCFA and essential oils against *C. perfringens*. The methodology selected for the in vitro testing provided a robust approach for the determination of interactions between the selected oils. In vivo the desing further provided a way to assess the efficacy of individual products and the combination to mitigate detrimental effects caused by necrotic enteritis challenge. The challenge model was robust resulting on clear affectation of the challenged control group, which aid in the comparison with the treatments. A limitation of this study could be the lack of an unchallenged control group, nevertheless other constraints (logistics, economic, spatial) gave priority to the desing with all groups challenged, unchallenged groups are particularly important when subclinical disease symptoms are achieve, however our study observed more severe response of the birds to the challenge model, which provides validity to the model and assessment of efficacy.

Chapter 4: GENERAL DISCUSSION

The current general discussion aims to complement the specific discussions included in each of the three manuscripts included in this thesis by focusing on broader aspects and mechanistic insights into the use of antibacterial plant combinations in relation to existing literature on similar strategies, particularly in the context of PWD caused by ETEC and ANE caused by *netB*+ *C. perfringens*. It begins with the *Hypotheses and Aims*, introducing the hypotheses behind each study, followed by the thesis approach, which describes the framework used to address these hypotheses. Next, the *Antibacterial Properties* of Individual Plants are discussed in comparison with available literature. In *The combination of plant materials*, the text explores the possible mechanisms for synergistic interactions. Finally, the section on *Beyond Antibacterial Action* examines additional effects of the strategies, such as their ability to modulate gut microbiota and improve overall gut health. The last part focuses on the combination used in the study with chickens.

Hypotheses, Aims and Thesis Approach

We hypothesize that dietary supplementation with a combination of plant-based antimicrobials can mitigate the severity of ETEC-induced PWD in weaning piglets and *netB*+ *C. perfringens*-induced necrotic enteritis in broiler chickens. **Study-I** and **Study-III** aimed to assess the efficacy of plant-based antimicrobial combinations targeting either ETEC or *netB*+ *C. perfringens* in controlled disease challenge experiments. **Study-II** further evaluated the impacts of dietary supplementation from **Study-I** on piglets three weeks after challenge and continuous dietary supplementation.

This thesis presented an approach to screening and testing alternative antimicrobials for combating gastrointestinal pathogenic diseases, focusing on four key aspects: (**1**) identification and screening of targeted antibacterial plants based on their antimicrobial properties against specific pathogens (in vitro), (**2**) investigation of their combined effects for enhanced antibacterial action (in vitro), (**3**) efficacy testing using disease challenge models (in vivo) and (**4**) assessment of their impact on the gut ecosystem beyond the acute phase of the challenge (in vivo). The use of targeted plant materials aims to minimize disruption of the beneficial gastrointestinal microbiota. Additionally, their combination seeks to leverage synergistic effects for enhanced antimicrobial efficacy, potentially reducing the inclusion levels of individual components and the risk of bacterial resistance. Furthermore, **Study-I** and **Study-II** used crude antibacterial plant materials directly as feed, whereas **Study-III** used semi-pure plant extracts. This comparison provides insight into the advantages and disadvantages of each strategy for using plant-based antibacterials.

Regarding the investigations focusing on ETEC and PWD, aspects (1) and (2) were examined by the MAFFRA-I and MAFFRA-II projects. In the frame of MAFFRA-I, twelve plant species were identified for their activity against ETEC. Ramsons (wild garlic) and acidic berries were the most effective in vitro, displaying synergistic antibacterial activity. Furthermore, in a pilot study, weaning pigs fed a mixture of ramsons and lingonberries (3% each) had reduced fecal and GIT *Enterobacteriaceae* levels without affecting lactic acid bacteria populations or feed intake (Canibe et al., 2018). The MAFFRA-II project supported aspects (1) and (2) by

providing in vitro antibacterial analyses of multiple garlic cultivars with different allicin concentrations, both alone and in combination with either apple pomace or blackcurrant. The selection and concentrations of plant materials used in **Study-I** and **Study-II** were based on these studies. **Study-I** dealt primarily with aspect (3), in a pathogenic challenge experiment with organic pigs challenged with ETEC-F18. **Study-II** focused on aspect (4) by investigating the effects of the plants supplementation 3 weeks after weaning. **Study-III** incorporated all aspects concerning *netB*+ *C. perfringens* and ANE.

The role of antibacterial plant combinations against ETEC

The antibacterial properties of fruits and berries

Previous work by our group (MAFFRA I project; unpublished data; Højberg et al., unpublished) investigated the antibacterial efficacy of acidic fruits, berries (e.g., lingonberries, redcurrants), and other antibacterial plants (ramsons, clove, horseradish) against ETEC in vitro. From these experiments, it was concluded that, in general, acidic berries and fruits could inhibit ETEC in the pig digesta incubation model (**Figure 4.1**). Plant materials that substantially reduced the pH compared to the control showed this antibacterial effect most prominently in experiments using various growth conditions (different digesta, concentrations, combinations; **Figure 4.1**). Indeed, it has been previously established that reducing the pH of gastrointestinal contents in vitro from 6.5 to 5.5 hinders the growth of pathogenic *E. coli* (Duncan et al., 2009).

Fruit acidity is primarily influenced by the accumulation of organic acids, which vary with species and cultivar (Bordonaba and Terry, 2010, Nour et al., 2011, Jiang et al., 2022, Golubkina et al., 2022). Citric acid is dominant in citrus fruits, currants, and rhubarb, while malic acid prevails in apples, pears, and peaches. Furthermore, the content of organic acids changes during fruit development and postharvest processing (Martău et al., 2021, Jiang et al., 2022). Due to their content of organic acids and other antimicrobial compounds, fruit and berries have been suggested to provide protection against gastrointestinal pathogens (Puupponen-Pimiä et al., 2005, Das et al., 2017).

Organic acids and their salts are used extensively in several industries as preservation agents primarily due to their antimicrobial properties (Hirshfield et al., 2003). Organic acids are also used for preservation of animal feeds (Tugnoli et al., 2020). Commonly used organic acids include butyric, formic, propionic, lactic, citric, acetic, fumaric, malic, and sorbic acids, often blended for consistent microbial control (Patience and Ramirez, 2022). Salts of organic acids are often preferred as they are typically odorless, less volatile, more soluble, and less corrosive than their pure acids counterparts (Tugnoli et al., 2020). For a while, it has been known that organic acids provide benefits for growth and health, particularly in young animals (Ravindran and Kornegay, 1993, Tugnoli et al., 2020, Patience and Ramirez, 2022). Their impact is influenced by type and dietary concentration, diet composition, and the characteristics of the animal (de Lange et al., 2010). In the past, most studies on organic acids have not specifically targeted ETEC or aimed to reduce diarrhea as the main endpoint (Canibe et al., 2022). However, their antibacterial action and influence on the gastrointestinal tract have laid the groundwork for their use in preventing PWD. Calcium formate and fumaric, citric, and formic acid can enhance growth performance, feed intake, and intestinal health while reducing diarrhea and pathogen shedding in weaning pigs challenged with ETEC (Lallès et al., 2009, de Lange et al., 2010, Tugnoli et al., 2020). On the other hand, concerns over safety, corrosiveness, alteration of gastric HCl secretions and development of bacterial acid resistance have been raised (Hirshfield et al.,

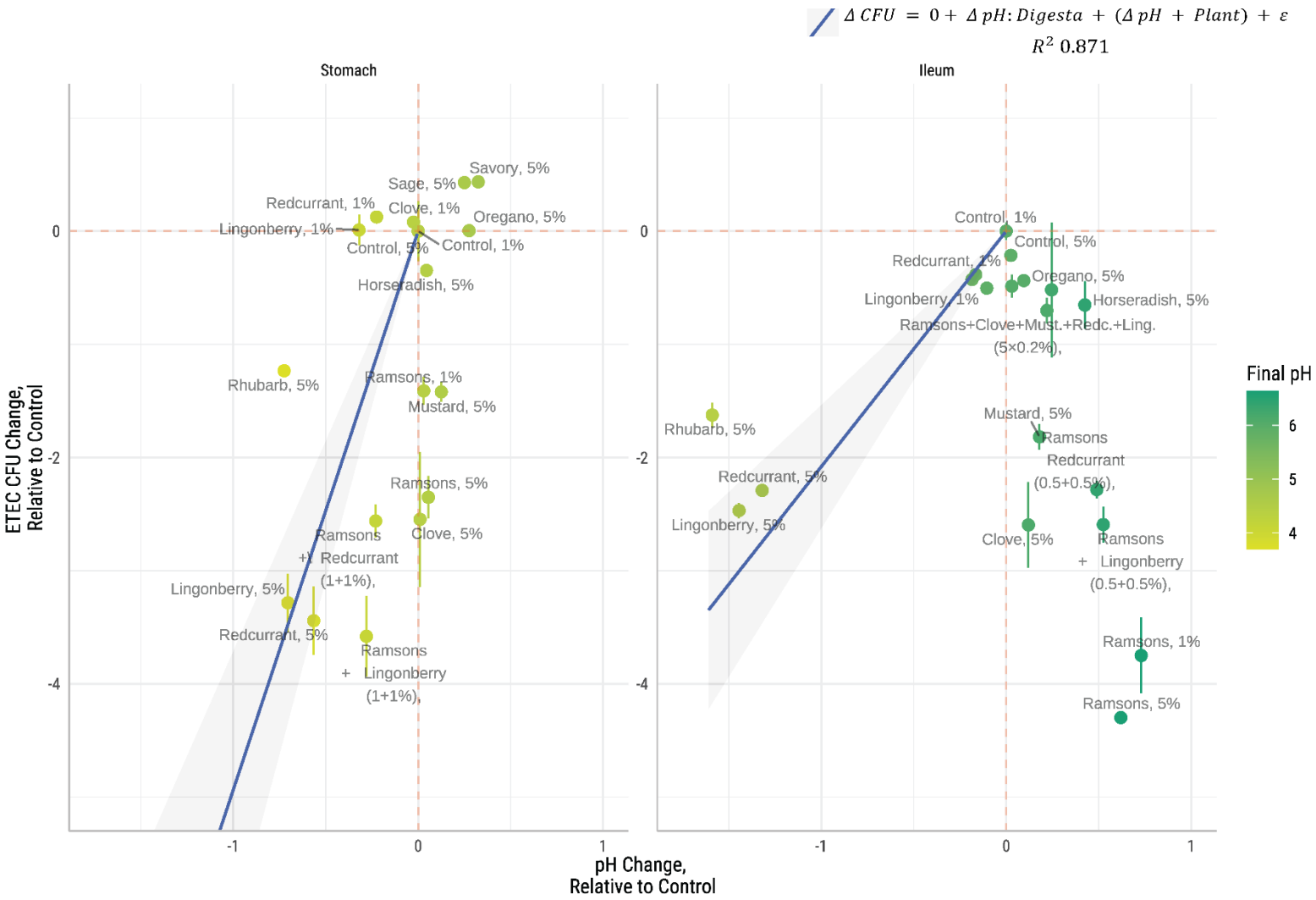


Figure 4.1 Relationship between ETEC F4 relative CFU change and pH relative change in experiments conducted with antibacterial plant material in vitro. Data from four in vitro experiments during the MAFFRA-I project utilized savory, sage, oregano, horseradish, rhubarb, mustard, ramsons, clove, lingonberry, and redcurrant. The experiments used different incubation digesta types (stomach and small intestine), plant concentrations and combinations. Changes in CFU and pH were normalized against control samples, thus the zero intercepts represent the relatively similar change against control. The blue line represents the prediction of a mixed model fit with relative pH change and digesta type as fixed effects and plant materials as random effects. The shaded area represents the 95% confidence interval for prediction.

2003, Tugnoli et al., 2020, Luise et al., 2020). Furthermore, the inclusion levels necessary to influence gastric pH and modulate microbial populations are typically above 1%, which in some instances may not be cost-effective (Tugnoli et al., 2020). Nevertheless, *Salmonella enterica* and *E. coli* challenged piglets fed 0.5% citric showed improved growth performance and reduced pathogenic bacterial loads compared to untreated controls (Ahmed et al., 2014).

The antibacterial properties of organic acids are linked to their permeability of cell membranes when undissociated (determined by environmental pH and individual pK_a), a mechanism known as anion model/anion stress (Russell and Diez-Gonzalez, 1997). The internal pH of *E. coli* (pH 7.6) is kept higher than external conditions by exporting protons via active transport (Duncan et al., 2009). Several organic acids (including SCFAs, but also lactic acid, benzoic acid and others) can freely cross the bacterial cell membranes (Moore et al., 2019). Once inside, the acids dissociate (H+ and RCOO–) in the cytoplasm and cannot longer cross the membrane, causing accumulation of anions and depression of internal pH (Ma et al., 2022). Bacterial pH regulation depletes energy resources, forces the bacteria to expel essential ions, overall affecting growth (Maurer et al., 2005, Moore et al., 2019). Furthermore, organic acids interfere with other *E. coli* cellular processes such as DNA replication and methionine synthesis, which is dependent on the anion permeating (Hirshfield et al., 2003, Ma et al., 2022).

It could be argued that similar benefits might be expected from natural sources of organic acids when incorporated into diets, including acidic berries, fruits, and their by-products. In **Study-I and -II**, apple pomace and blackcurrant were included aiming to provide acidifying properties using readily available and cost-effective plant materials. Blackcurrant was chosen for its high acidity (pH 2.81), while apple pomace, although slightly less acidic (pH 3.28), was selected for its favorable characteristics, availability in scale, and potential to provide further use for an otherwise waste byproduct.

Although varying multifactorially, the average content of organic acids in blackcurrant is about 200 mg/g DM (Bordonaba and Terry, 2008, Nour et al., 2011) *versus* about 30-40 mg/g DM in apple pomace (Waldbauer et al., 2017, Antonic et al., 2020b, Martău et al., 2021). Using this information, it could be estimated that the provision of organic acids in the diets (3% inclusion) was about 6 mg/g in the GB diet and about 0.1 mg/g in the GA diet. As mentioned earlier, it has been suggested that providing pure individual organic acids at 1% (10 mg/g) in diets is necessary to achieve the observed modulatory effects, highlighting the role of synergies in our combinations of plant materials.

The role of the content of organic acids of cloudberry, raspberry and blackcurrant in their antibacterial activity against *E. coli* and nonvirulent *S. enterica* has been previously demonstrated (Puupponen-Pimiä et al., 2001, Puupponen-Pimiä et al., 2005, Nohynek et al., 2006). The antibacterial effects are primarily observed between pH 3 and 5, which may be related to the pK_a of the organic acids present in the berries (Puupponen-Pimiä et al., 2005). Furthermore, complementary antibacterial effects may arise from the interaction between organic acids and other berry components, such as tannins. This is supported by Nohynek et al. (2006), who observed destabilization of the outer membrane in organic acid-free extracts of the same berries.

In **Study-I**, we observed that the dietary inclusion of garlic and apple pomace (**GA**) or garlic and blackcurrant (**GB**) was able to reduce the incidence of PWD in piglets challenged with ETEC F18. The reduction in PWD was accompanied by a decrease in the fecal shedding of

ETEC F18. These findings indicated that the plant-based combinations effectively inhibited the growth and colonization of ETEC F18 in the piglet's gut, thereby reducing clinical signs of PWD. The pH of the apple and blackcurrant powders, as well as the acid-binding capacity (ABC-4) of the experimental diets, were measured to confirm their acidifying properties. The results showed that the blackcurrant powder had a lower pH than the apple pomace powder, and the diet containing blackcurrant had a lower ABC-4 than the diet containing apple pomace. These findings support the rationale for selecting these specific plant materials based on their pH-lowering potential.

Intriguingly, previous studies incorporating fruits, berries, and pomace into piglet diets have not primarily targeted organic acid provision but have instead emphasized the delivery of fermentable dietary fibers and antioxidant polyphenols. Sehm et al. (2007) provided diets containing 3.5% apple pomace to weaning piglets for 50 days. Although Sehm and collaborators did not see changes in growth performance compared to an untreated control, the pigs receiving apple pomace supplementation had improved intestinal morphology and reduced gut-associated lymphoid tissue activation via ileum Peyer's patches (Sehm et al., 2006, Sehm et al., 2007). More recently, Dufourny et al. (2021) reported a positive influence of apple pomace supplementation (2 and 4 %) on performance parameters and modulation of the cecal and fecal microbiota.

Interestingly, in Dufourny et al. (2022), *Catenibacterium* was more abundant in pigs receiving 4% apple pomace, which coincided with our observation of increased abundance of this genus in fecal, cecal and colonic microbiota of pigs in **Study-I** and **Study-II** receiving GA or GB. In contrast, Dufourny et al. (2022) observed a tendency for reduced fecal consistency and increased pathogen excretion, which is in opposition to our observations in **Study-I**. However, their study did not involve an ETEC challenge, and apple pomace was provided individually. Additionally, the content of organic acids, pH, and acid-binding capacity were not evaluated, complicating comparison with the conditions in our study.

In **Study-I**, both combinations reduced diarrheal symptoms and ETEC shedding, the GB treatment seemed more effective antibacterial. In contrast, in **Study-II**, the GA treatment had relatively more influence on the GIT ecosystem. These differences could be driven by the individual composition of the combinations, i.e., garlic with either apple pomace or blackcurrant. These plant materials contain an array of phytochemicals with potential bioactive properties as presented in Chapter 1. Indeed, preliminary results from an untargeted metabolomics screening of the plant materials used in **Study-I** and **-II**, showed a clear PCA separation between the blackcurrant and apple pomace sample (Figure 4.2). The separation is driven by compounds characteristic of each plant material, for example quercitin and D-glucuronic acid in the apple pomace cluster, whereas citrate and rutin drove the blackcurrant separation.

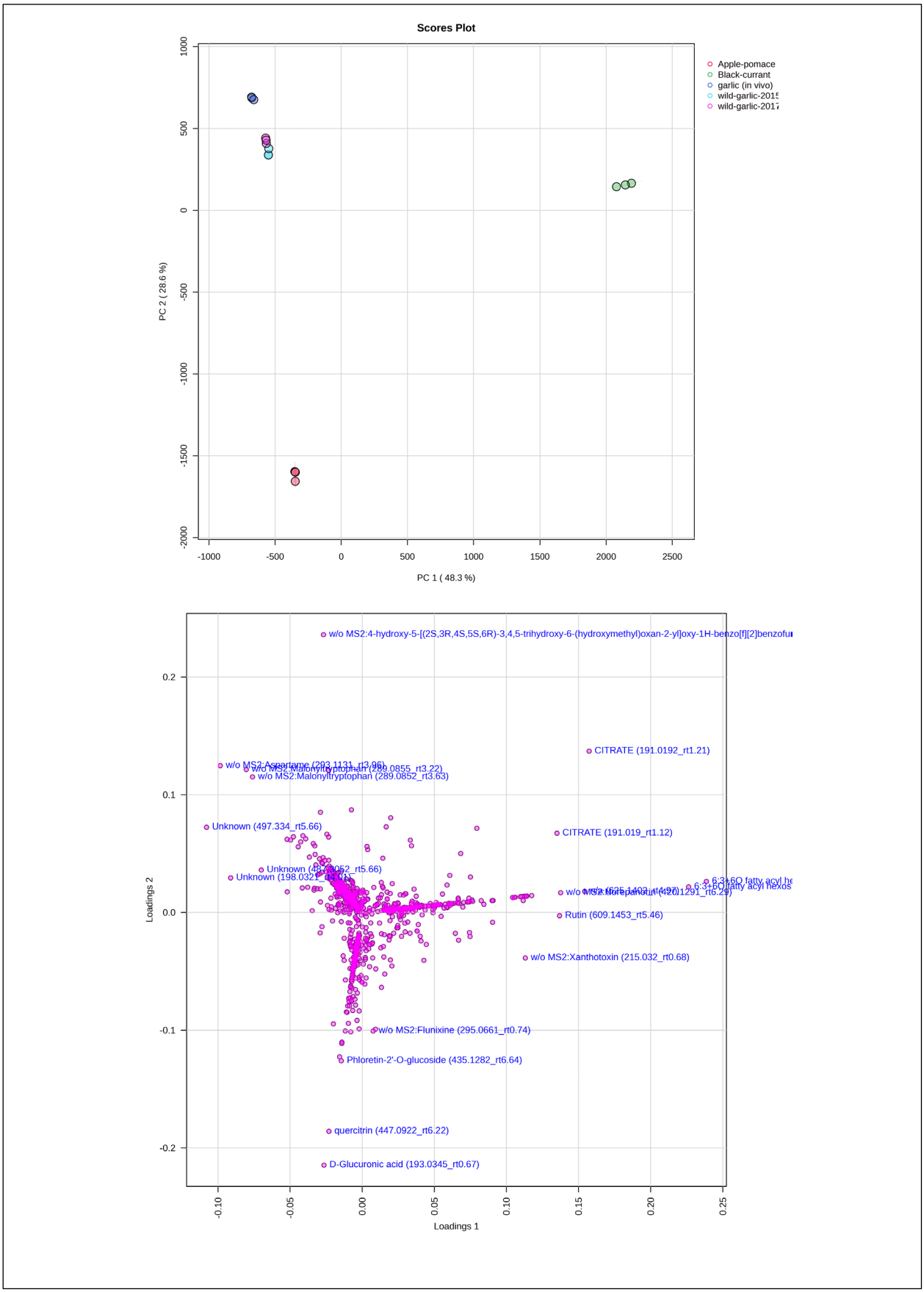


Figure 4.2 Principal component analysis of preliminary metabolomic analysis of plant materials used in Study I and Study II. Garlic, apple pomace, blackcurrant powders. Wild garlic powder used in MAFFRA-I project are also presented. The top figure is the PCA plot, while bottom figure is loadings plot showing metabolites that contribute to separations.

The antibacterial properties of garlic

The antibacterial properties of garlic are expected to primarily come from allicin and other organosulfurs as presented in Chapter 1. A limitation for comparison of studies involving garlic (and other plant materials) is the lack of reported information regarding active components in the specific batches used. Moreover, the variety of garlic products, which contain different compounds (Chapter 1; Garlic), further complicates the direct comparisons. Table 4.1 summarizes the concentration of garlic-derived active components reported on recent studies using garlic in weaning pigs.

Table 4.1 Concentration of garlic phytochemicals in products used in studies with weaning piglets. Reported active compound concentration and calculated phytochemical provision compared to allicin content in garlic used for Study-I and -II.

Garlic product	Active compound concentration in product (Reported)	Inclusion level	Calculated active provision	Reference
Garlic powder	Allicin: 44 mg/g	3% feed	140 mg/100g feed	Study-I, Study-II
Garlic botanical (steamed distillation oil)	Propyl thiosulfonates: 40%	10 ppm feed	0.4 mg/100g feed	Liu, 2013
Fermented garlic	Alliin[13]: 750 mg/kg	2 g/kg feed	0.15 mg/100g feed	Yan, 2013; Wang, 2011
Garlic powder	Allicin: 5 mg/g	0.3 g/kg BW	7.2 – 8.1 mg/pig/day	Ayrle, 2019
Purple garlic powder	Alliin /100g feed	2 % feed	14 mg/100g feed	Rivera-Gomis, 2020; Serrano-Jara, 2023

The studies by Liu et al. (2013) investigated the supplementation of garlic botanical extract (40% propyl thiosulfonates, steamed distillation oil,10 ppm feed) to ETEC-F18 challenged piglets (Liu et al., 2013, Liu et al., 2014). Compared to an untreated challenged control, the supplementation reduced diarrhea scores and frequency, and white blood cell counts, while increasing ileal villi height (Liu et al., 2013). However, Liu et al. (2013) did not observe an influence of the treatment on growth performance or fecal ETEC shedding. The latter findings contrast with our findings in **Study-I**, where improved performance and reduced ETEC shedding was observed. The reason for the discrepancy could be different quantity and characteristics in active ingredients. For comparison, in Liu and collaborators, the provision of propyl thiosulfonates would be of around 0.4 mg/100g feed, whereas the provision of allicin in **Study-I and -II** was of approximately 140 mg/100g feed (Allicin in garlic powder: 44 mg/g, 30 g/kg feed). Moreover, propyl thiosulfonates—degradation products of allicin—exhibit approximately half the antibacterial potency of allicin (Ruiz et al., 2010, Sorlozano-Puerto et al., 2018). However, their greater stability often makes them the preferred choice for standardized preparations (Ruiz et

[13] Alliin itself is not antibacterial as mentioned in Chapter 1.

al., 2010, Sorlozano-Puerto et al., 2018).

In the studies of Yan and Kim (2013) and Wang et al. (2011) using fermented garlic in piglets challenged with LPS or non-challenged, they observed improved growth performance, immune response and reduced fecal shedding of *E. coli*. Indeed, the authors attributed the positive impact on growth performance to the reduced *E. coli* counts. Both studies used the same plant material, which was characterized to have an alliin content of 750 mg/kg (about 7 times lower than the garlic cloves they used for the fermentation), equivalent to 0.15 mg/100g feed (when fermented garlic was added at 2 g/kg feed). Although these authors attributed the reduction in *E. coli* to antibacterial effects from the allicin in garlic, this seems unlikely given the fact that the preparation of the fermentation included a crushing step, and water addition, which would mean rapid formation and possibly degradation of allicin (reflected in the reported alliin content).

Yan and Kim (2013) and Wang et al. (2011) further reported the measured content of DADS and DATS in the fermented garlic, which were 4.40 mg/kg and 32.8 mg/kg, respectively. It could be speculated that the antibacterial effects of the degradation products inhibited ETEC, but the concentrations were rather low. Potential synergistic interactions with products formed during the fermentation process could also offer insights; however, these were not reported. Interestingly, in their study with unchallenged animals, although the *E. coli* numbers decreased linearly with increasing concentrations of fermented garlic (0.5, 1, 2 g/kg), growth performance was enhanced only when using the lowest concentration, contrary to their observations with challenged animals (Yan and Kim, 2013). A common observation with feed additives and other alternatives to antibiotics is that benefits are often only seen in challenged animals, not those under normal conditions, making the strategies less attractive to producers.

Recent studies with a larger number of animals under commercial farm conditions have shown contrasting results. Ayrle et al. (2019) reported improved ADG and clinical health, but no significant reduction of diarrhea nor fecal coliforms counts in pigs receiving garlic powder (5 mg/g allicin) topped in their feed ration. The feed intake was not reported in this study, but based on their report, the provision of allicin would be 7.2 – 8.1 mg of allicin per pig per day (garlic powder topped on feed 0.3 g/kg BW daily). For comparison, the pigs on **Study-I** would have an intake of about 500 mg allicin/per pig per day the first week of the experiment, assuming about 400 g ADFI. The provision of garlic powder topped on feed may also present challenges as strong odor may lead to intake problems (and difficult handling), but the authors did not report the feed intake.

Another group of studies using purple garlic powder in unchallenged piglets and under commercial conditions reported improved growth performance and improved intestinal morphology (Rivera-Gomis et al., 2020, Serrano-Jara et al., 2023a, Serrano-Jara et al., 2023b). The authors did not report the allicin content of the powder; however, they measured the organosulfur content of the diets before and after the experiment. In the diets with an inclusion of 20 g purple garlic powder/kg, the resulting alliin content was about14 mg/100g feed. Moreover, most of the organosulfur in their diets was in the form γ-Glutamyl-S-allyl-cysteine (about 180 mg/100g feed). γ-Glutamyl-S-allyl-cysteine is a water-soluble peptide intermediate to the formation of S-alk(en)yl-L-cysteine sulfoxide, with no antibacterial properties itself, but with antioxidant properties (Tan et al., 2015). In their study, the cecal counts of *E. coli* were not different to those of untreated controls (Serrano-Jara et al., 2023a). An important observation

from these studies with purple garlic was the reduction of total organosulfur compounds over time, as measured in the feed at the start and end of each diet phase (day 1 *versus* day 15, and day 1 *versus* day 34) (Serrano-Jara et al., 2023b). This underscores the natural degradation of garlic's active components, which is an important consideration when incorporating garlic products into diets.

Overall, most of the reported studies using garlic products in piglets' experiments are unable to observe antibacterial effects, which might not be surprising as it seems that the concentrations of active components may have been too low to induce any antibacterial activity. In contrast, in our studies (**Study-I** and **Study-II**), the garlic powder was conceived to provide antibacterial effect from the selection of a high allicin garlic cultivar, careful preparation of garlic powder and confirmation of high allicin content before incorporation to the diet.

The combination of plant materials

Citric acid accounts for about 85% of the organic acids in blackcurrant, followed by about 6.5% ascorbic and 6.5% malic acid, and less than 2% tartaric and oxalic (Bordonaba and Terry, 2008, Nour et al., 2011). On the other hand, in apple pomace, malic acid accounts for about 45%, followed by citric acid and oxalic acids, with about 25% each, and less than 5% ascorbic acid (Martău et al., 2021). Different organic acids have varying effectiveness against pathogenic *E. coli*, which may be related to their ability to cause anion stress. For example, Kim and Rhee (2013) observed a greater antibacterial activity of malic (pK_a 3.4) and lactic acid (pK_a 3.86) in comparison to citric (pK_a 3.13) and acetic acid (pK_a 4.75) against Shiga toxin-producing *E. coli*. Organic acids with lower pK_a are more likely to dissociate (at physiological pH) thus unable to permeate the bacterial cells and cause anion stress (Nguyen et al., 2020), but have a higher capacity to reduce the pH, also resulting in growth inhibition for organisms like *E. coli*. For this reason, combinations of organic acids are preferred as differing pK_a values give the chance for combine actions and synergism (Nguyen et al., 2020, Patience and Ramirez, 2022, Ji et al., 2023). Indeed, combinations of organic acids are added at relatively low levels (<1%) in diets (Nguyen et al., 2020).

Furthermore, the synergistic action of organic acids with other substances has been documented before. Organic acids with lower pK_a values demonstrated greater bactericidal activity when combined with MCFAs (which as mentioned in Chapter 2 are antibacterial by disrupting cell membranes). For example, in the aforementioned study by Kim and Rhee (2013), the authors observed that although citric acid was not the most antibacterial against pathogenic *E. coli*, when combined with caprylic acid, the resulting damage to the cell membrane allowed hydrogen ions to pass into the cell, causing a marked bactericidal effect. This property has been replicated in studies with pigs with varying success. Zentek et al. (2013) reported that a combination of organic acids (0.416% fumaric and 0.328% lactic acid) with MCFAs (0.15% caprylic and 0.15% capric acid) reduced the pH along the GIT and *E. coli* virulence genes, although with no influence on growth. Lei et al. (2017) observed reduced diarrhea and increased growth performance in ETEC-challenged pigs receiving a blend of fumaric acid, citric acid, malic, capric and caprylic acid, however, bacterial counts were not measured. Similar effects have been seen in other studies combining different organic acids with capric acid and caprylic acid (Nguyen et al., 2020).

A similar mechanism could have played a role in the results observed in **Study-I**, which is further supported by the in vitro observations (Figure 4.1; MAFFRA I project; unpublished data;

Højberg et al., unpublished). The combination of organic acids (malic, oxalic, and citric acids) and other substances (e.g., polyphenols) from apple pomace and blackcurrant, in combination with the antibacterial properties of garlic, could provide an enhanced antibacterial effect against ETEC. Allicin and other organosulfur compounds from garlic can act as cell membrane permeabilizers (Li et al., 2020), which, when combined with organic acids like citric acid, could facilitate the entry of these acids into bacterial cells, disrupting internal pH homeostasis and enhancing their antibacterial action. Moreover, phenolic acids also act as weak acids permeating bacterial membranes and causing anionic stress (Alibi et al., 2021). An antibacterial strategy based on complementary effects could be of more relevance when targeting pathogenic *E. coli*. For example, several enteropathogenic *E. coli* strains have developed acid resistance withstanding gastric pH (1.3-3) and even anion stress caused by SCFA (Li et al., 2020).

Another example of a similar mechanism for synergism in antimicrobial combinations has been suggested for the enhancement of antimicrobial activity of antibiotics when combined with garlic, which has been documented in vitro, particularly against MDR bacteria (Herman and Herman, 2023). This involves the alteration in the structure and integrity of the membranes, facilitating the uptake and subsequent effectiveness of the tested antibiotics (Cai et al., 2007, Magryś et al., 2021). The synergistic interactions of different and complementary mode of action contribute to not only increase efficiency on utilization (less product needed for activity), but also provide further benefits as reducing risk of bacteria developing resistance to the strategy used, and "restoring" antimicrobial effects from products that have lost potency due to AMR development (Cheesman et al., 2017).

Combination strategies have become more popular in recent years, with many proprietary formulations entering the market, although active ingredients and concentrations are often not disclosed in the literature (Patience and Ramirez, 2022). Wong et al. (2022) recently investigated the combination of capsicum oleoresin and garlic oil (two types; synthetic and steam distilled, details not specified) on weaned pigs infected with ETEC-F18. The same researchers previously investigated the individual effects of capsicum oleoresin, and a similar garlic extract in (Liu et al., 2013). Similar to their earlier studies, they observed that the combinations used at the same concentration resulted in improved intestinal morphology, reduced diarrhea frequency, and modulated immune responses (Wong et al., 2022). However, these effects were not observed at lower doses, indicating a lack of complementary effects. Likewise, they did not observe changes in growth performance or ETEC fecal shedding (Wong et al., 2022). Although the active ingredient composition of the plant materials used was not reported, it is likely that the composition was similar to their previous report (Liu et al., 2013, Liu et al., 2014, Wong et al., 2022). Thus, it was likely insufficient to display antibacterial activity, in contrast to our observations in **Study-I**. An interesting observation from their research indicates that even without direct antibacterial action, there can still be a reduction in diarrhea and an improvement in intestinal morphology. This suggests resistance to the detrimental effects of enterotoxins in the animals fed the garlic oil used by Liu et al. (2013), (Liu et al., 2014) and Wong et al. (2022).

Although not necessarily involving garlic and fruits/berries, other examples exist in the literature where the synergistic action of organic acids along with antibacterial agents is exploited (Patience and Ramirez, 2022). However, most studies report improvements in either physiological parameters or growth performance, while antibacterial effects are seldom achieved (Canibe et al., 2022). For example, a blend of organic acids (fumaric, malic, citric, and

sorbic acids) and essential oils (thymol, vanillin, and eugenol) improved intestinal morphology and reduced diarrhea in piglets challenged with ETEC-F4, but did not affect cecal copy counts of the *faeG* gene (F4 fimbriae) (Choi et al., 2020a). Similar responses have been observed in other studies involving combinations of organic acids and essential oils (Jiménez et al., 2020, Xu et al., 2020). In most of these studies, the inclusion level of the treatments was below 1g/kg.

Overall, studies combining plant-based antibacterial substances often report improved physiological responses, reduced diarrhea, and occasional performance enhancements, even without direct antibacterial effects. In contrast, in **Study-I**, we observed antibacterial action, likely due to synergistic effects determined in previous in vitro research (Figure 4.1; MAFFRA I project; unpublished data; Højberg et al., unpublished). However, further studies should refine the combinations that can optimize efficacy, particularly focusing on the most active components (e.g., allicin and organic acid concentrations).

Beyond antibacterial action

In addition to the antibacterial effects primarily observed in **Study-I**, our results further indicated the ability of the plant materials to modulate the gut microbiota in **Study-II**. The provided plant materials have a high content of readily fermentable substances. Indeed, as discussed earlier, supplementation of apple pomace to pigs has been done with the aim of providing fermentable fibers, in addition to apple polyphenols (Sehm et al., 2007, Pieszka et al., 2017, Dufourny et al., 2019, Dufourny et al., 2021, Dufourny et al., 2022). Furthermore, garlic powder also contains an important quantity of polysaccharides in the form of fructans, which account for approximately 26–30% of fresh weight (Zhao et al., 2022). In **Study-I** and **-II**, the level of supplementation translated in about 15 g/kg of fructans in the diet. Previous studies with piglets have shown that supplementation of fructans (~1 g/kg) decreases the diarrhea symptoms and *E. coli* counts while increasing *Lactobacillus* counts (Adewole et al., 2016). Fructans, being readily fermentable, can lower luminal pH and reduce the populations of pH-sensitive enteropathogenic bacteria (Knudsen, 2019). In contrast, previous studies using chicory root in piglets similar to the ones used in **Study-I** (organic-weaned), reported no effect of fructans (chicory root) supplementation against *E. coli* challenge (Hedemann and Bach Knudsen, 2010). Although in **Study-II**, we did not observe differences in the pH along the GIT, we observed positive modulation of bacterial genera associated with fermentation in the cecum and colon.

Other authors have pointed at the content of fructose in apple pomace as having trophic effects in the GIT, reflected in improved intestinal morphology (Jang et al., 2018, Dufourny et al., 2021). We did not measure intestinal morphology parameters, but further studies could evaluate the impact of the plant combinations on this aspect as we observed a slight increase in the fructose content in the diets containing the plant combinations (**Study-I**).

The role of antibacterial plant combinations against ANE

In **Study-III**, the combination of palm kernel fatty acids (PKFAs) and clove essential oil proved synergistic antibacterial activity against *netB+ C. perfringens* in vitro. Interestingly, a similar mechanism to that observed with the combinations of garlic and acidic berries could have played a role here. PKFAs, rich in lauric acid, disrupt bacterial cell membranes, while eugenol in clove oil permeabilizes membranes and inhibits essential enzymes (Skřivanová et al., 2005, Gill and Holley, 2006). The combined action of these substances can lead to enhanced disruption of bacterial cells and increased antimicrobial efficacy.

As mentioned above, the combination of organic acids (including MCFAs) and essential oils is a strategy for enhanced antibacterial effects. Pham et al. (2022) used a combination of a commercial blend containing 4% carvacrol, 4% thyme, 0.5% caproic (C6), 3.5% benzoic, and 0.5 % butyric acid, in dosages of 0.2, 0.5, and 0.8 g/kg feed to chickens under necrotic enteritis challenge. They saw improved growth performance, reduced intestinal lesions, and *C. perfringens* load in the caecum and liver of the ANE-challenged chickens, accompanied by increased relative abundance of *Lactobacillus* (Pham et al., 2022). Despite the low provision of active substances, the authors observed an antibacterial action from the blend. It should be noted that in the study of Pham et al. (2022), the ANE model was similar to the one used in **Study-III**, except for the provision of fish meal, which they did not include in their model, probably explaining the comparatively milder symptoms observed in their study.

Another study, using a combination of MCFAs (1.7 g/kg; capric-caprylic; caproic and lauric acid), essential oil components (2 g/kg; cinnamaldehyde, carvacrol, and thymol) and organic acids (1.5 g/kg calcium butyrate + fumaric and citric acid) in ANE-challenged chickens improved the growth performance, intestinal structure, and reduced *C. perfringens* counts, although only at the end of the experiment (Abdelli et al., 2020). In the experiment by Abdelli and colleagues, although the chickens were subjected to an ANE challenge model and did not exhibit clinical signs of ANE, they observed significant growth depression, which they interpreted as a sign of infection (Abdelli et al., 2020). Their ANE model used recycled litter from a previously infected flock for disease reproduction (Abdelli et al., 2020).

Other studies have been reported using combinations of multiple organic acids, MCFAs and essential oils in chickens, but not using ANE challenges (Abd El-Hack et al., 2022). Another challenge for comparison of studies is the lack of reporting of concentrations of active ingredients.

Chapter 5: CONCLUSIONS AND PERSPECTIVES

General Conclusion

The combination of targeted antibacterial plants provides an effective strategy for the control of enteric diseases in piglets and broilers. In the studies, part of the current PhD thesis, we were able to develop a framework for the screening, selection, and testing of plant materials to target specific gastrointestinal pathogens. Furthermore, we identified plant combinations that demonstrated enhanced antimicrobial efficacy. These combinations not only reduced pathogen load but also promoted beneficial gut microbiota (particularly in the pig studies) and supported overall health and growth performance in piglets and broilers.

In our studies with pigs, we showed that selected plants could provide antibacterial properties comparable to those of conventional feed additives. The use of crude plant materials for functional properties may be preferred over more processed compounds, particularly in organic systems. Consumer preferences may also favor products with fewer "artificial" compounds. Furthermore, minimally processed plant materials face fewer restrictions for use, not only in organic systems but also in conventional pig production, where feed additive policies impose intricate rules on product approval.

The inclusion levels and provision of active antimicrobial agents in the plant materials seem to have played a significant role in the observed antibacterial effects, particularly in the case of the combinations against ETEC. On the other hand, the provision of complete plant materials may have also elicited the chance for further benefits besides the antibacterial effects, reflected in the positive modulation of the microbiota and GIT ecosystem. We further showed that a relatively high provision of antibacterial compounds in the plant material was safe and generally positive for animal health and performance.

Synergistic combinations of plant-based antimicrobials offer several advantages. They can reduce the required dosage of individual supplements, minimizing potential adverse effects and cost while maintaining or even enhancing their effectiveness. Additionally, synergistic combinations can target multiple bacterial species and virulence factors, providing broader protection against various diseases, and likely offering less risk of development of antimicrobial resistance.Our findings highlight the potential of plant-based antimicrobial combinations as a sustainable alternative to antibiotics in monogastric livestock production, paving the way for further research and practical applications in the field.

Perspectives

Future studies on the use of garlic and acidic berries, fruits, or co-products should focus on refining the concentrations needed to achieve effective antibacterial action. Additionally, since our current research involved organically weaned animals, it is important to evaluate whether animals weaned at a younger age could similarly benefit from these plant material supplements under challenge conditions. For the combination used against chicken ANE, the need for scaling and optimization also applies. Future studies could investigate the role of combining polyphenolic compounds and eugenol on the quorum sensing mechanisms of *netB*+ *C. perfringens*. Since quorum sensing plays a crucial role in the pathogenesis of this disease, developing methods to manipulate this system could provide additional tools to prevent disease onset.

More complex formulations (3 or more plants) with complex plant mixtures could be further studied, as different mechanisms of action may enhance efficacy against pathogens. Furthermore, the investigation of synergistic activities provides more attractive and more cost-effective strategies. However, care should be taken as interactions could also result in antagonistic effects. Thus, the present work could provide a framework for the study of combinations of antibacterials for use in animal studies. Further interactions or complementarity effects of plant materials with other strategies could be evaluated in the future, for example the provision of plant materials along probiotics could further provide benefits from the strategy.

Given the differential organoleptic properties provided by these plant materials, it would be valuable to assess feed preference in pigs. Since feed intake is a critical challenge at weaning, the enhanced attractiveness of feed containing the proposed combination of plant materials could offer a significant advantage. Furthermore, evaluating these benefits in larger cohorts of pigs is necessary to assess the impact on mortality rates and to better understand the benefit-cost balance of this strategy.

Assessing the economic viability of antibacterial plants material is crucial for optimizing production costs and fostering a sustainable industry. This involves in-depth research to understand the financial implications of sourcing, processing, and distributing the plant materials. Additionally, ensuring a stable and local supply chain is vital to avoid reliance on imported materials, ease price fluctuations, and support local economies. A comprehensive analysis should also consider the environmental impact of increased production, including land use competition with other industries and potential effects on biodiversity. Finally, the analysis of competition of the utilization in animal feed *versus* utilization in other industries also warrants investigation.

Ensuring antibacterial quality is crucial for consistent implementation. Therefore, the development of commercial strategies for utilizing these plants should focus on standardizing product quality. Furthermore, identifying key active compounds and their synergistic combinations within effective plant materials to aid in standardization. For example, standardization can be achieved by combining batches to maintain a minimum level of active compound concentration. Given the natural variability of active components in plant materials, another approach could involve the synthetic production of these key compounds, providing a tool for developing more standardized products. This focus could enable the creation of reproducible antibacterial formulations, potentially reducing reliance on conventional antimicrobials. Additionally, it is important to highlight the limitations of organic schemes, necessitating more research on minimally processed plant materials.

An important aspect not explored in this work, but offering major potential for future research, is the prevalence of ETEC infections in humans. These infections primarily impact travelers and children in developing countries, often caused by MDR strains. The insights gained from studying plant-based antimicrobials in animal models could potentially translate to novel treatments for human ETEC infections, especially those caused by MDR strains.

In general, the use of multi-omics approaches could also provide insights on effects of the plant materials on the GIT ecosystem, the interplay with the microbiota, the host metabolism and immune responses, and the nature of the synergies observed, further providing mechanistic insights. This could be propelled by advances in data science and artificial intelligence which could make sense of complex data pools.

STUDY-I

Antibacterial Plant Combinations Prevent Postweaning Diarrhea in Organically Raised Piglets Challenged with Enterotoxigenic *Escherichia coli* F18

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Antibacterial plant combinations prevent postweaning diarrhea in organically raised piglets challenged with enterotoxigenic *Escherichia coli* F18

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Antibiotics and zinc oxide restrictions encourage the search for alternatives to combat intestinal pathogens, including enterotoxigenic *Escherichia coli* (ETEC), a major cause of postweaning diarrhea (PWD) in pigs. PWD causes important economic losses for conventional and organic farming. This study investigated the effect of dietary supplementation with garlic and apple pomace or blackcurrant on infection indicators and the fecal microbiota of organic-raised piglets challenged with ETEC-F18. For 21 days, 32 piglets (7-weeks-old) were randomly assigned to one of four groups: non-challenge (NC); ETEC-challenged (PC); ETEC-challenged receiving garlic and apple pomace (3 + 3%; GA); ETEC-challenged receiving garlic and blackcurrant (3 + 3%; GB). ETEC-F18 was administered (8 mL; 10^9 CFU/ml) on days 1 and 2 postweaning. The 1st week, PC had lower average daily gain than those in the NC, GA, and GB groups ($P < 0.05$). NC pigs showed neither ETEC-F18 shedding nor signs of diarrhea. The PC group had higher diarrhea incidence and lower fecal dry matter than NC (≈5–10 days; 95% sEBCI). The GA and GB groups showed reduced ETEC-F18 and *fedA* gene shedding, higher fecal dry matter, and lower diarrhea incidence than the PC (≈5–9 days; 95% sEBCI). The NC, GA, and GB had normal hematology values during most of the study, whereas the PC had increased ($P < 0.05$) red blood cells, hemoglobin, and hematocrit on day 7. Haptoglobin and pig-MAP increased in all groups, peaking on day 7, but PC showed the greatest increase ($P < 0.05$). The fecal microbiota of PC pigs had reduced α-diversity (day 7; $P < 0.05$) and higher volatility (days 3–14; $P < 0.05$). *Escherichia*, *Campylobacter*, and *Erysipelothrix* were more abundant in the PC than in the NC, GB, and GA groups ($\log_2$FC > 2; $P < 0.05$), whereas *Catenibacterium*, *Dialister*, and *Mitsoukella* were more abundant in the NC, GB, and GA than in the PC group ($\log_2$FC > 2; $P < 0.05$). *Prevotella* and *Lactobacillus* were more abundant in the GB group ($\log_2$FC > 2, $P < 0.05$). In conclusion, dietary supplementation of GA and GB limited ETEC proliferation, reduced PWD, and beneficially impacted the fecal microbiota's diversity, composition, and stability.

KEYWORDS

antibacterial plant, apple pomace, blackcurrant, diarrhea, *Escherichia coli*, garlic, microbiota, organic pigs

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) expressing F4 and F18 fimbriae is recognized as the main causative agents of postweaning diarrhea (PWD). It is a substantial cause of morbidity and mortality in young pigs, resulting in economic loss due to the cost of treatment, impaired growth, and animal loss (1, 2).

Antibiotics and medical levels of zinc oxide have long been used to treat and prevent PWD in conventionally produced pigs. However, antimicrobial resistance and environmental issues now limit their use (3). Since 2006, the European Union has banned all antibiotic growth promoters and has recently adopted new regulations limiting the amount of zinc in animal feed to 150 ppm and restricting any form of routine antibiotic use (4). Alternative strategies and/or additives are therefore needed to sustainably reduce the need for antibiotic use while promoting pig health, growth, and welfare.

The prevalence of PWD is also a challenge in organic farming systems, where the use of antibiotics and other synthetic drugs is restricted (5). Based on prescription data for Danish organic pig production in 2016, Kruse et al. (6) reported that 65% of antimicrobial treatment doses in weaners were prescribed for gastrointestinal diseases, accounting for the majority of antibiotic usage on these farms. High pH and reduced stomach emptying in piglets after weaning have been linked to pathogen proliferation and PWD (7). The proliferation and attachment of ETEC to the pig intestinal mucosa is a crucial step in the pathogenesis of PWD (1). Hence, dietary strategies that selectively diminish ETEC growth or limit adhesion to enterocytes, or potentially both, are crucial for reducing PWD (7).

Medicinal plants and related products have been suggested as prophylactic and therapeutic alternatives against gastrointestinal diseases in pigs (8). Indeed, previous research from our group indicated that supplementing piglet diets with *Allium ursinum* (wild garlic) bulbs and *Vaccinium vitis-idaea* (lingonberries) elicits antibacterial effects against coliforms along the gastrointestinal tract, while having no effect on lactic acid bacteria counts (9). The bactericidal effect was attributed to the allicin in wild garlic bulbs and the pH-lowering effect of the berries; additionally, *in vitro* screening revealed that these effects were synergistic (9). However, because the supply of wild garlic bulbs and lingonberries is limited (harvested from nature and thus expensive plant resources), it is relevant to determine whether similar effects can be obtained with a combination of more readily available and less expensive plant material with comparable properties.

Garlic (*Allium sativum*) is the most studied plant of the *Allium* species in terms of organosulfur compound concentrations (10). Allicin is formed from garlic and garlic powders when the enzyme, alliinase, is activated by crushing cloves or wetting powder, allowing alliin to be converted to allicin and other allyl thiosulfinates (11). Apple pomace, a byproduct of mashing and pressing apples (*Malus domestica*) for juice, cider, or puree, has been identified as a valuable source of polyphenols, dietary fiber, and organic acids (12, 13). Apple pomace has been reported to have a pH $\approx$ 4 (12, 14), varying by cultivar and maturity of apples at harvest. Blackcurrant (*Ribes nigrum*) is a perennial shrub native to central Europe and northern Asia (15) that is cultivated in gardens and berry orchards for juice production and has been shown to inhibit *E. coli* (16). Blackcurrants contain high levels of pectic polysaccharides, anthocyanins, and organic acids. The pH of blackcurrants is $\approx$ 2.7 (17), but this varies depending on the cultivar.

As observed by Canibe et al. (9) with the mixture of wild garlic and lingonberries, the combined antibacterial effect of allicin from garlic and the acidifying properties of apple pomace or blackcurrant, are expected to inhibit ETEC proliferation in the gut of weaned piglets. We hypothesized that supplementing piglets with garlic and apple pomace, or garlic and blackcurrant alleviates clinical signs of PWD by selectively targeting ETEC and promoting a healthy gut microbiota. The objective of this study was to assess infection indicators and fecal microbiota dynamics in organically reared weaned piglets following a postweaning ETEC F18 challenge and dietary supplementation with garlic powder in combination with apple pomace or blackcurrant powder.

2. Materials and methods

The study was performed at the Department of Animal and Veterinary Sciences of Aarhus University (Denmark). The animal and experimental procedures were approved by the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food Administration (License 2017-15-0201-01270). Animal care and housing were in accordance with Danish laws and regulations governing the humane care and use of animals in research.

2.1. Antibacterial plant preparation

Fresh organically cultivated garlic (Therador cultivar) was obtained from Årslev Research Center (Department of Food Science, Aarhus University, Denmark), and a small amount from imported from GIE l'Ail Drômois (Eurre, France). The garlic cloves were cut into chips using a cutter (AK-RAMON; Talleres Ramon, S.L., Barcelona, Spain) and dried in shallow layers at 40°C in a ventilated oven (Binder GmbH, Tuttlingen, Germany) before being ground into a fine powder (Ø = 0–1 mm) in a centrifugal mill (ZM200; Retsch GmbH, Haan, Germany). The garlic powder was stored in sealed plastic bags at −20°C until it was used. This preparation of the dry powder guaranteed the presence of alliin and alliinase, which upon wetting results in enzymatic allicin production (i.e., allicin is generated when pigs ingest the powder). To confirm activity, the allicin produced by the garlic powder upon wetting was determined. Samples were analyzed using high-pressure liquid chromatography (HPLC). Briefly, a 50 mg sample was incubated in water (5 ml, MilliQ) at room temperature for 10 min to allow the alliinase enzyme to convert alliin to allicin. Following sedimentation, 1 ml supernatant from each sample was mixed with 1 ml methanol (HPLC grade) and then centrifuged for 3 min at 10,621 g and filtered through a 0.4 µm nylon filter into HPLC vials. A standard curve (R^2 = 0.995) to quantify the potential allicin content expressed as mg alliin Eq/g dry powder was created by spiking different known amounts of alliin (S-Allyl-L-cysteine sulfoxide, 74264; SigmaAldrich, Søborg, Denmark) against

a standard garlic sample. This method of quantification (18) was used since reference compounds of pure allicin are not available due to low stability. Measurement was performed on a Nexera X2 LC30AD HPLC system (Shimadzu, Kyoto, Japan) equipped with an SPDM20A prominence diode array detector and a Hypersil GOLD column (4.6 × 250 mm; 5 μm; Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Apple pomace from juice pressing was obtained from Orskov Foods (Ørbæk, Denmark). The apples were mainly of the cultivar Elstar and cultivated organically in their own orchard. The apple pomace was immediately frozen after pressing and stored at −20°C until use. The frozen apple pomace was thawed and ground in a wet grinder (GM300, Retsch GmbH) to ensure that the seeds were macerated, allowing for possible enzymatic cyanide formation, before drying to evaporate any accumulated cyanide. Drying was done on shelves in a Memmert drying cabinet at 60°C during 5 days. Dry apple mash was milled into a fine powder (Ø = 0–1 mm) using a centrifugal mill (ZM200, Retsch GmbH). The powder was stored in sealed plastic bags at −20°C until use. Freeze dried blackcurrant (unspecified cultivar) powder (Ø = 0–1 mm) was derived from organic berries grown in Lithuania, and provided by the company Berrifine (Ringsted, Denmark). The pH of the apple and blackcurrant powders was tested in a 25% (w/v) Milli-Q water solution.

Various concentrations of the powders were tested alone or in combination for their ability to kill or inhibit the growth of ETEC in porcine gastric digesta (9). Based on the expected concentration of the plant combinations in the stomach upon consumption, the results from the *in vitro* testing were used to determine the appropriate inclusion level of garlic, apple pomace, and blackcurrant powders in the feed (data not shown).

2.2. Animals, experimental design, and diets

Thirty-two piglets (7-weeks-old; body weight = 20.14 ± 1.5 kg) born from four organically raised sows (TN70 Topigs; Norsvin, Esbjerg Ø, Danmark) were selected for the study (eight piglets per sow, mixed sex). The sows were confirmed to be homozygote carriers of the dominant gene (*FUT1*^{GG}) encoding ETEC F18 fimbriae receptors by VHL genetics (Wageningen, The Netherlands); thus, piglets were genetically susceptible to ETEC F18 (19). The pigs were obtained at weaning and the study lasted 21 days. The weaned piglets were housed in pens of two littermates. There was no physical contact between pigs housed in different pens. Treatment groups were housed in different rooms (similar in design) to avoid cross contamination. The rooms were climate-controlled (20 ± 0.4°C).

Two piglets, housed in the same pen, from each sow were randomly assigned to one of four treatments: non-challenged control fed a standard diet (Negative Control, NC); ETEC-challenged control fed a standard diet (Positive Control, PC); ETEC-challenged fed the standard diet supplemented with garlic and apple pomace (3 + 3% w/w, GA); and ETEC-challenged fed the standard diet supplemented with garlic and blackcurrant (3 + 3% w/w, GB). Treatments were balanced by initial body weight.

The diets followed the guidelines for organic pig production and were formulated to meet the Danish nutrient requirement standards of pigs (20). The ingredient composition is presented in Supplementary Table 1.

2.3. Challenge with enterotoxigenic *Escherichia coli* and sampling procedures

The ETEC strain (O138 F18-ETEC 9910297-2STM) expressing F18 fimbriae, heat-stable enterotoxin b (STb); heat-labile enterotoxin (LT); enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1), and Shiga toxin type 2e (Stx2e), was isolated from the intestinal content of a pig with PWD and was provided by the Danish Veterinary Institute (Copenhagen, Denmark). The ETEC F18 was found to be hemolytic (on blood agar). Enterotoxigenic *E. coli* F18 colonies from blood agar plates were transferred to BHI broth and incubated at 37°C for 5 h with constant shaking (150 rpm). Following centrifugation (12,000 rpm, 10 min), the bacteria were diluted in 0.9% NaCl and the bacterial load was adjusted to OD_{600} = 1.0, corresponding to 10^9 CFU/ml. The concentrations of the bacterial suspension were confirmed by plate count (blood agar). On days 1 and 2 after weaning, the PC, GA, and GB pigs were orally challenged with 7–8 ml of 0.9% NaCl solution containing ≈10^9 CFU/ml of ETEC F18. The NC pigs were given 7–8 mL of 0.9% NaCl orally. To administer the solutions, a polyethylene tube was connected to a syringe and placed in the mouth of the piglet.

The pigs were monitored daily for signs of illness, such as diarrhea, lethargy, and dehydration. Individual body weight was recorded at weaning, and on days 7, 14, and 21 postweaning to estimate average daily gain (ADG) and daily feed disappearance per pen was recorded for calculation of average daily feed intake (ADFI). Feed efficiency was then estimated as weight gain to feed intake ratio (G:F).

Fecal samples were collected on the weaning day, daily for the 1st week, and three times a week for the last 2 weeks of the study. The fecal samples were scored on a 7-point scale for consistency (1: hard, dry and cloddy; 2: firm; 3: soft but able to retain some shape; 4: soft and unable to retain any shape; 5: watery and dark; 6: watery and yellow; 7: foamy and yellow) with scores 4 to 7 defined as clinical signs of diarrhea (21). Samples were kept on ice until they were divided into three aliquots for genomic analyses (−80°C), dry matter (−20°C), and ETEC F18 enumeration (conducted immediately).

Individual blood samples were collected from the jugular vein at weaning, and days 3, 5, 7, 14, and 21 postweaning (1, 3, 5, 12, and 19 days after second infection) in EDTA-containing collection tubes for hematology analysis, and heparinized collection tubes for determination of pig C-reactive protein (CRP), haptoglobin, and pig major acute-phase protein (pigMAP) concentrations. Hematology parameters were analyzed immediately after collection. Plasma was obtained from heparinized tubes that were stored on ice and centrifuged (2,000 × *g*, 10 min) within 2 h following collection. Plasma was stored at −20°C until acute phase proteins were analyzed.

2.4. Analytical methods

The acid-binding capacity at pH 4 (ABC-4) of the diets was determined as described by Lawlor et al. (22). Dry matter (DM) of diets was determined by drying to constant weight at 103°C (≈20 h), ash according to method 923.03 AOAC (23). Gross energy was measured on a 6300 Automatic Isoperibol Calorimeter system (Parr Instruments, Moline, IL, USA). The crude fat was determined by HCl-Bligh and Dyer extraction as described by Jensen (24). Crude protein (N × 6.25) was determined by the Dumas method (25). Amino acids in diets were determined using the European Economic Community methods (26). The dietary content of sugars (glucose, fructose and sucrose), fructans, starch, soluble and insoluble non-starch polysaccharides, and Klason lignin were measured as described in Knudsen (27).

The dry matter content of the feces (F-DM) was determined by freeze-drying the samples to a constant weight. To determine ETEC counts, ∼1–3 g of feces were suspended in a peptone solution (1:10, w/v) and homogenized with a bag blender (Smasher; Biomérieux, Marcy-l'Étoile, France). Serial 10-fold dilutions were prepared and 100-μL aliquots were spread-plated on blood agar (Columbia blood agar with sheep blood medium, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated aerobically overnight at 37°C. Hemolytic bacteria were counted using a manual colony counter and the count expressed as CFU/g feces. Blood agar plates with hemolytic colonies were stored at 5°C until ETEC F18 serotyping was performed using the slide agglutination test with type-specific antisera (SSI Diagnostica A/S, Hillerød, Denmark) on five colonies per plate. The limits of detection were 10^4 CFU/g on days 1 and 11–21, and 10^5 CFU/g feces on days 2–9; the higher detection limit was applied on days where the counts were likely to increase.

Quantitative polymerase chain reaction (qPCR) was used for quantification of the gene encoding the F18 fimbriae (*fedA* gene) and STb toxin (*estB* gene) in fecal samples. Briefly, DNA was extracted from fecal samples using the NucleoSpin 96 DNA Stool kit (Macherey-Nagel, Düren, Germany). The concentration of genomic dsDNA was measured using the Qubit Broad Range Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) on an Invitrogen Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Following extraction, qPCR was performed on a ViiA 7 real-time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) using a MicroAmp Optical 384 well reaction plate (Applied Biosystems, Waltham, Massachusetts, USA). The qPCR reactions contained 5 μL of Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA), the F18 and STb primers at a concentration of 0.3 mM, 2 μL of template DNA and water to a final volume of 10 μL. All samples were analyzed in triplicate and the data generated in QuantStudio real-time PCR Software v1·4. Primer sequences and qPCR specific settings are presented in Supplementary Table 2.

Complete blood counts were determined using a ProCyte Dx Hematology analyzer (IDEXX B.V., Hoofddorp, Netherlands). Blood plasma haptoglobin was determined using the PHASE Haptoglobin Assay Kit (TP801; Tridelta Developments Ltd., Kildare, Ireland). The CRP and Pig-MAP were determined by particle enhanced immune turbidimetry, using the Turbovet pig CRP and Turbovet Pig-MAP kits (Acuvet Biotech, Zaragoza, Spain). The determinations were conducted using an ADVIA 1800 Clinical Chemistry System autoanalyzer (Siemens Healthineers AG, Erlangen, Germany).

2.5. Fecal microbiota profiling

Fecal samples from days 1, 3, 7, 14, and 21 postweaning were used for microbiota profiling. Total microbial DNA was extracted from the samples as previously stated for qPCR analyses and used for bacterial profiling using 16S rRNA gene amplification. Only samples with a DNA concentration >7 ng/μl were used for PCR amplification and for paired-end sequencing of the 16S rRNA V3-V4 region (250-bp paired-end raw reads) on the Illumina platform NovaSeq 6000 (Illumina, San Diego, CA, United States). Library preparation, DNA quality control (Agarose Gel Electrophoresis; 5400 Fragment Analyzer, Agilent, USA) and sequencing was performed by Novogene (Novogene UK Company Limited, Cambridge, United Kingdom).

The raw amplicon sequencing data was processed into the table of exact amplicon sequence variants (ASV) using the DADA2 pipeline (28) in R (version 4.2) for the identification of ASVs. Reads were filtered and trimmed based on quality, de-noised, and merged, then chimeras removed, and taxonomy was assigned to each ASV using a naive Bayesian classifier method against the SILVA reference database v138 (29). Subsequent filters included the removal of read lengths shorter than 398, non-bacterial and cyanobacteria reads, and ASVs with prevalence <0.005 across all samples.

Analyses were performed after rarefying at 90% of minimum sampling depth, except for α-diversity measures, where unrarefied reads were used. The α-diversity (observed, Shannon, and inverse Simpson) and β-diversity measures (weighted UniFrac dissimilarity matrices) were calculated using phyloseq (30) in R (version 4.2.1). To assess community structure changes over time, microbiota volatility (31) was measured as the weighted UniFrac distance between the individual community structure from the previous individual timepoint. Differential abundance analysis was conducted using the DEseq2 package (32) in R (version 4.2.1) and involved a threshold of 0.1% relative abundance and 10% prevalence.

2.6. Statistical analyses

Differences in pig growth performance were assessed using ANOVA. The model included treatment as a fixed effect, initial body weight was included as a covariate for ADG and ADFI, and pen and sow effects were included as random effects (PROC MIXED, SAS Studio). Assumptions of normality and homogeneity of variance were confirmed. *P*-values were adjusted for multiple comparisons using the Holm-Bonferroni adjustment.

The remaining statistical analyses were carried out in R (version 4.2.1). Unless otherwise specified, *P*-values were adjusted for

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TABLE 1 Analyzed composition of experimental diets (dry matter basis).

Item	Diets[a]		
	NC—PC	GA	GB
Moisture, %	10.64	10.12	9.57
Crude protein, %	21.40	21.77	21.21
Gross energy, kcal/kg	4,476	4,456	4,451
Crude fat, %	4.06	4.15	3.79
Ash, %	4.96	5.07	5.20
Indispensable amino acids, %			
Arginine	1.25	1.23	1.21
Histidine	0.48	0.48	0.46
Isoleucine	0.92	0.92	0.88
Leucine	1.59	1.57	1.52
Lysine	1.36	1.35	1.33
Methionine	0.40	0.38	0.38
Phenylalanine	1.02	1.00	0.97
Threonine	0.83	0.83	0.80
Valine	1.13	1.12	1.08
Dispensable amino acids, %			
Alanine	0.98	0.97	0.94
Aspartic acid	1.86	1.87	1.78
Cysteine	0.36	0.34	0.34
Glutamic acid	3.70	3.64	3.59
Glycine	1.00	0.98	0.96
Proline	1.29	1.28	1.25
Serine	1.02	1.00	0.98
Carbohydrates, %			
Fructose	0.11	0.81	0.78
Glucose	0.25	0.77	0.53
Sucrose	2.74	3.12	3.02
Fructans	0.04	1.61	1.45
Starch	43.44	44.23	44.16
S-NSP[b]	3.57	2.35	3.60
Rhamnose	0.02	0.03	0.04
Fucose	0.01	0.01	0.02
Arabinose	0.59	0.51	0.67
Xylose	0.90	0.16	0.51
Mannose	0.20	0.18	0.22
Galactose	0.29	0.31	0.35
Glucose	1.27	0.65	1.36
Uronic acids	0.28	0.49	0.43
I-NSP[c]	11.73	11.88	12.13
Rhamnose	0.02	0.02	0.02
Fucose	0.02	0.02	0.03
Arabinose	1.73	1.63	1.61
Xylose	3.91	3.95	3.86
Mannose	0.26	0.38	0.30
Galactose	0.32	0.33	0.37
Glucose	1.23	1.83	1.31
Uronic acids	0.46	0.49	0.47
Cellulose	3.77	3.25	4.16
Total NSP[d]	15.29	14.23	15.73
Klason lignin	3.00	3.41	2.79
Dietary fiber[e]	18.33	19.25	19.96

[a]NC, non-challenge, standard diet; PC, challenged, standard diet; GA, challenged, garlic and apple pomace supplementation (3 + 3%); GB, challenged, garlic and blackcurrant supplementation (3 + 3%).
[b]Soluble non-starch polysaccharides.
[c]Insoluble non-starch polysaccharides.
[d]Total non-starch polysaccharides (S-NSP + I-NSP).
[e]Total non-starch polysaccharides + lignin + fructans.

multiple comparisons using the Benjamini-Hochberg method and differences were considered significant if $P < 0.05$.

Hierarchical generalized additive models (33) were used to analyze F-DM, fecal scores, ETEC counts, and qPCR data using the mgcv package (34). Fecal scores were modeled using ordered categorial distribution. Model diagnostics were assessed *via* the appraise function of the gratia package (35). The models included treatment as parametric term, independent smooths over time for each treatment, interaction smooths over time for each pig, and random effects of pen and sow. Pairwise differences between treatment groups against the PC group were assessed *via* 95% simultaneous empirical Bayesian confidence intervals (sEBCI) as described by Mundo et al. (36).

Results of acute phase proteins, and microbiota α-diversity measures and volatility were analyzed using generalized linear mixed models with day, treatment and their interaction as fixed effects, and random slopes by subjects, pen and sow were also included as random effects. The models were fit using the glmmTMB package, adequate distribution and covariance structure were selected by best fit and regression diagnostics using the performance package (37).

Microbiota β-diversity was examined by non-metric multidimensional scaling (NMDS) ordination and compared using permutational multivariate analysis of variance (PERMANOVA) in the vegan package (38). Differentially abundant taxa between treatments were identified using a time course experiment strategy in the DeSeq2 workflow (32). Briefly, a likelihood ratio test with a model including day, treatment, and their interaction was performed. Wald test was then used to evaluate taxa in pairwise comparisons against the PC group within day. The threshold for classification as differentially abundant taxa was $\log_2$ fold-change

TABLE 2 Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and dietary supplementation with plant combinations on postweaning performance of organic weaners.

Item	Treatment[d]				SEM[e]	*P*-value
	NC	PC	GA	GB		
Body weight, kg						
Weaning	20.17	19.98	20.58	19.81	1.451	0.987
Day 7	23.29	22.19	22.79	22.55	1.842	0.976
Day 14	28.32	27.78	27.30	26.92	2.132	0.966
Day 21	34.11	33.82	32.39	31.74	2.797	0.902
Average daily gain, g/d						
Day 0–7	441.3[a]	234.8[b]	432.3[a]	395.3[ab]	43.085	0.007
Day 7–14	714.5[ab]	840.1[a]	675.9[b]	628.3[b]	33.933	0.012
Day 14–21	823.5	841.7	781.8	692.6	55.745	0.486
Overall	660.9	633.1	607.4	569.8	48.542	0.333
Feed intake, g/d						
Day 0–7	793.1	784.7	660.6	746.2	35.79	0.141
Day 7–14	1,417	1,429.5	1,377.6	1,245.9	87.61	0.303
Day 14–21	1,742	1,757.1	1,560.3	1,589.9	75.45	0.169
Overall	1,318.7	1,281.9	1,200.3	1,156.7	61.44	0.291
G:F[f]						
Day 0–7	0.56[b]	0.35[c]	0.72[a]	0.53[b]	0.049	<0.001
Day 7–14	0.51	0.53	0.52	0.51	0.016	0.946
Day 14–21	0.47	0.48	0.5	0.46	0.015	0.809
Overall	0.5	0.48	0.51	0.49	0.017	0.736

[abc]Within a row, values that do not share a common superscript differ ($P < 0.05$), Holm-Bonferroni adjustment.
[d]NC, non-challenge ($n = 8$), standard diet; PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).
[e]Pooled standard error of least squared means.
[f]Gain to feed ratio (feed efficiency).

($\log_2$FC) > 2 and adjusted $P < 0.05$. The differentially abundant taxa were clustered using the pheatmap package.

3. Results

The potential allicin content in the garlic powder was found to be 44.1 ± 6.0 mg alliin Eq/g dry powder ($n = 3$). The pH of the apple pomace powder was 3.28 ± 0.01 and the pH of the blackcurrant powder was 2.81 ± 0.01. The initial pH (before ABC-4 measurement) of the Control (NC and PC), GA and GB diets were 6.39 ± 0.01, 6.16 ± 0.02, and 6.03 ± 0.01, respectively (n = 3). The ABC-4 of the Control (NC and PC), GA, and GB diets were 454.1, 388.8, and 323.2 mEq, respectively. The chemical composition of the GA and GB experimental diets was similar to that of the diet fed to NC and PC pigs (Table 1). The GA and GB diets had a greater content of fructans than the control diet i.e., 1.61 and 1.45 vs. 0.04%, respectively.

All pigs started the study in good health. The NC pigs were in good health throughout the study, whereas one pig from the PC group died 5 days postweaning and two pigs from the GA group in the same pen died on days 6 and 7, respectively. The deceased pigs had high counts of ETEC in the feces ($>10^8$ CFU/g feces; $>10^5$ *fedA* gene copies/g feces) and displayed signs of diarrhea (Fecal score = 6; <10% F-DM).

3.1. Growth performance

There was no overall effect ($P > 0.05$) of treatment on body weight, ADG, ADFI and G:F during the experiment (Table 2). During the 1st week after weaning, the PC pigs had lower ADG than NC pigs, but no difference in ADFI was detected between treatments, resulting in PC having the lowest G:F of all treatments. When compared to the PC group, the GA and GB pigs had higher ADG in the 1st week. In contrast, the PC group had the highest ADG during the following week. The ADG and ADFI of the GA and GB pigs did not differ from those of the NC pigs during any of the calculated periods. During the 1st week of the study, the GA pigs had the highest G:F, followed by NC and GB and then finally PC.

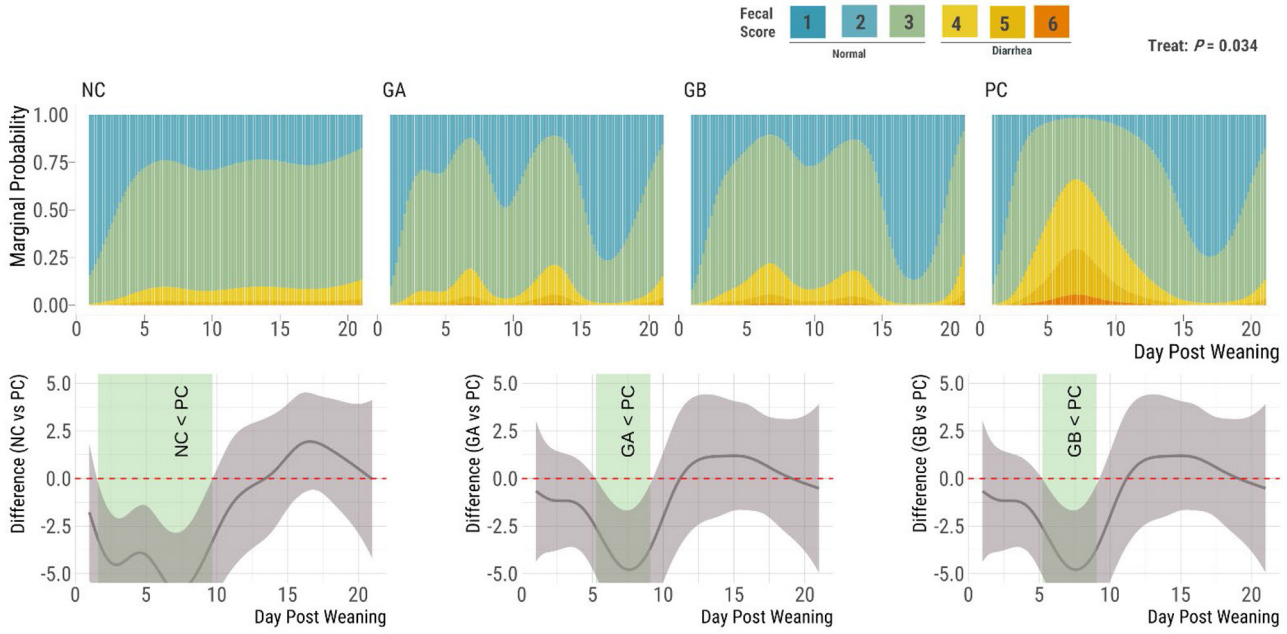

FIGURE 1
Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and dietary supplementation with plant combinations on fecal consistency scores in organic weaner pigs (based on a 7-point scale with scores 4–7 indicating diarrhea). The ETEC F18 was administered orally on days 1 and 2. **(Top panel)** Stacked bar plots for the marginal probability occurrence of each score category over time by treatment. **(Bottom panel)** Pairwise comparisons against PC; significant differences exist where the 95% empirical Bayesian simultaneous confidence interval does not cover 0 (green shade). NC, non-challenge, standard diet ($n = 8$); PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).

3.2. Fecal consistency and fecal dry matter

The fecal consistency score was influenced by treatment ($P = 0.034$; Figure 1). The cumulative marginal probability of loose stools (scores 4–7) was below 15% overall in the NC pigs, below 20% for the GA and GB groups, and above 50% in the PC group between day ≈5 and 9. The NC group had a higher (95% sEBCI) proportion of firm feces (and fewer soft feces) than the PC group from days 5 to 10, while the GA and GB groups had a higher (95% sEBCI) proportion of firm feces than the PC group from days 6 to 8 postweaning.

The F-DM was influenced by treatment ($P = 0.018$; Figure 2). The mean F-DM percentage for the NC, GA, and GB groups remained above 20% throughout the study, while the PC pigs had the lowest percentages of F-DM from 5 to 9 days postweaning, dropping below 20%. The F-DM of the NC pigs was higher (95% sEBCI) than that of the PC groups from days 5 to 10. When compared to the PC group the GA and GB pigs had higher (95% sEBCI) F-DM from days 5 to 9 postweaning.

3.3. Fecal shedding of enterotoxigenic *Escherichia coli* F18 and virulence factor genes

The fecal counts of ETEC F18 were influenced by treatment ($P < 0.001$; Figure 3). There was no detectable ETEC F18 in the feces of pigs prior to ETEC challenge. During the study, the ETEC F18 counts in the feces of the NC pigs were around the detection limits; whereas counts in the other groups were detectable from days 2 to 11 and remained stable at low levels thereafter. The ETEC counts in feces of NC pigs were lower (95% sEBCI) than in the PC pigs from days 2 to 9. When compared to the PC group, the GA group had lower counts (95% sEBCI) from days 4 to 7, and the GB group had lower counts from days 4 to 8 postweaning.

The *fedA* gene was not detectable in the feces of the pigs prior to ETEC challenge, and the copy number was influenced by treatment thereafter ($P < 0.001$; Figure 4). The F18-encoding gene was not present in the NC pigs at any time point during the study. Gene copies increased in all challenged groups from day 2, but the PC group had the highest mean levels of the *fedA* gene. The NC pigs had fewer *fedA* gene copies (95% sEBCI) than the PC pigs from days 2 to 11. When compared to the PC group, the GA and GB groups had fewer (95% sEBCI) gene copies from days 6 to 9 postweaning.

The *est-II* gene (STb toxin) was detectable in all groups prior to ETEC challenge (Figure 5). The shedding of the gene in feces increased in all groups after weaning and differed (95% sEBCI) among groups from days 2 to 7. The NC group had the lowest (95% sEBCI) levels of gene copies during the whole study period. Gene copies increased in all groups beginning on day 2, but the PC group had the highest mean (95% sEBCI) levels of *est-II* gene. The NC pigs shed fewer (95% sEBCI) copies of the gene than the PC pigs from days 2 to 9. When compared to the PC group, the GA pigs shed fewer STb-encoding gene copies from days 5 to 9 postweaning (95% sEBCI), and the GB pigs from days 5 to 8 postweaning (95% sEBCI).

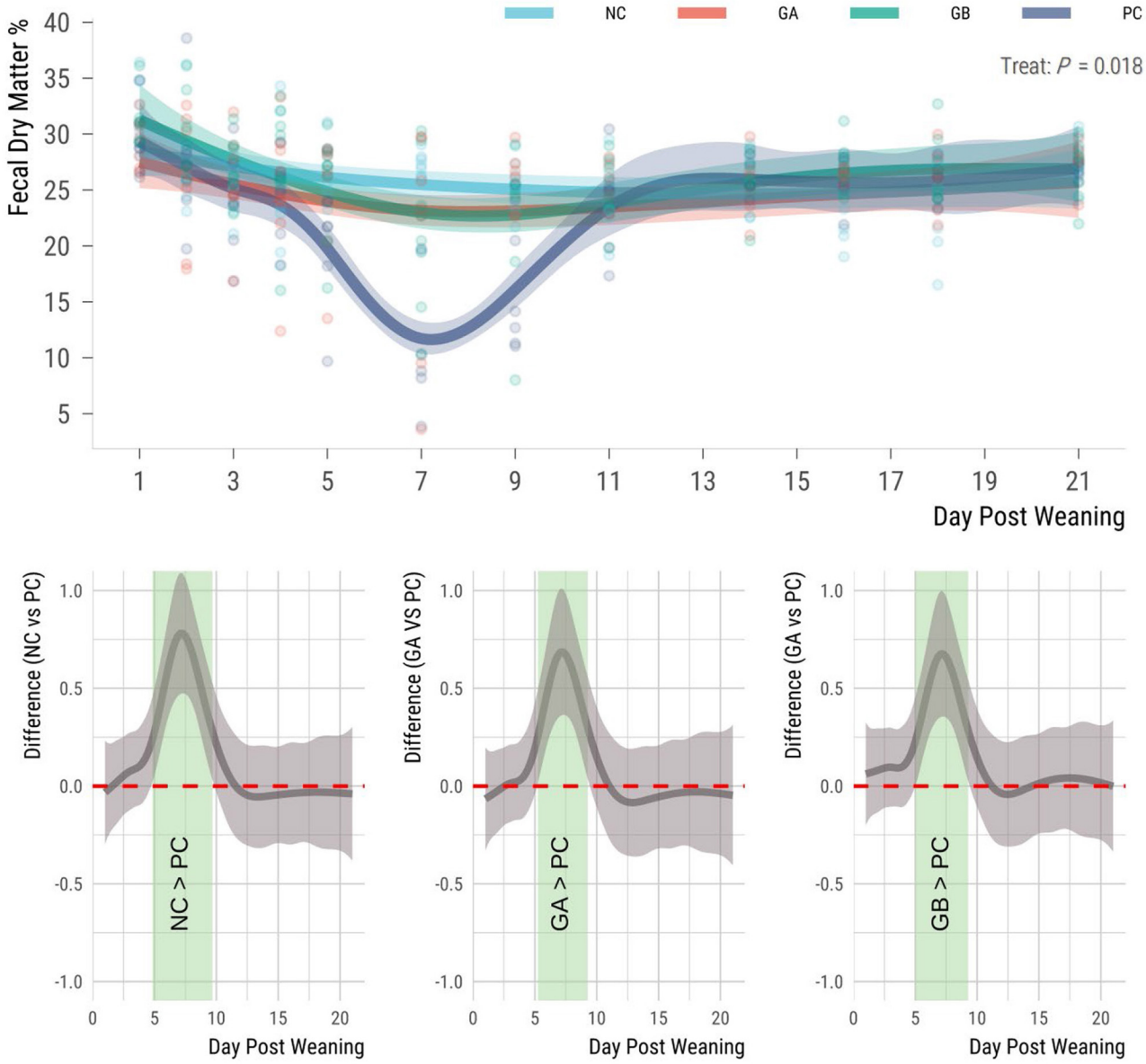

FIGURE 2
Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and dietary supplementation with plant combinations on fecal dry matter (%) of organically reared weaner pigs. **(Top panel)** Lines represent trends for each group; shaded regions are 95% confidence intervals. The ETEC F18 was administered orally on days 1 and 2. **(Bottom panel)** Pairwise comparisons against PC; significant differences exist where the 95% empirical Bayesian simultaneous confidence interval does not cover 0 (green shade). NC, non-challenge, standard diet ($n = 8$); PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).

3.4. Blood hematology

The effect of treatment on the red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell value (MCV), mean cell hemoglobin (MCH), and reticulocyte count was dependent on the day ($P < 0.001$; Table 3). The mean cell hemoglobin content (MCHC) and platelet count were unaffected by the treatments. On the 1st day of the study the GB pigs had lower HCT and reticulocyte count than the NC pigs. By day 3, the reticulocyte count in GA group was less than the NC and PC pigs, and the GB pigs had a reduced reticulocyte count than the NC group. By day 7, the RBC, HBC, and HCT were lower levels in NC, GA, and GB pigs than in PC group. On day 14, HGB levels were reduced in GA and GB pigs, and their reticulocyte count was higher than in NC and PC pigs. Finally, on day 21, the GA and GB pigs had higher reticulocyte counts and reduced RBC, HGB, and HCT levels than the NC and PC pigs, while the MCV in GA pigs was higher than in NC and PC groups.

The white blood cell (WBC) count, and the neutrophil, and basophil proportions were not influenced by treatment (Table 4). The interaction between day and treatment influenced the proportion of lymphocytes, monocytes, and eosinophils. On the 1st day of the study, the monocytes proportion of the NC, GA, and GB groups was higher than that of the PC group. On day 3,

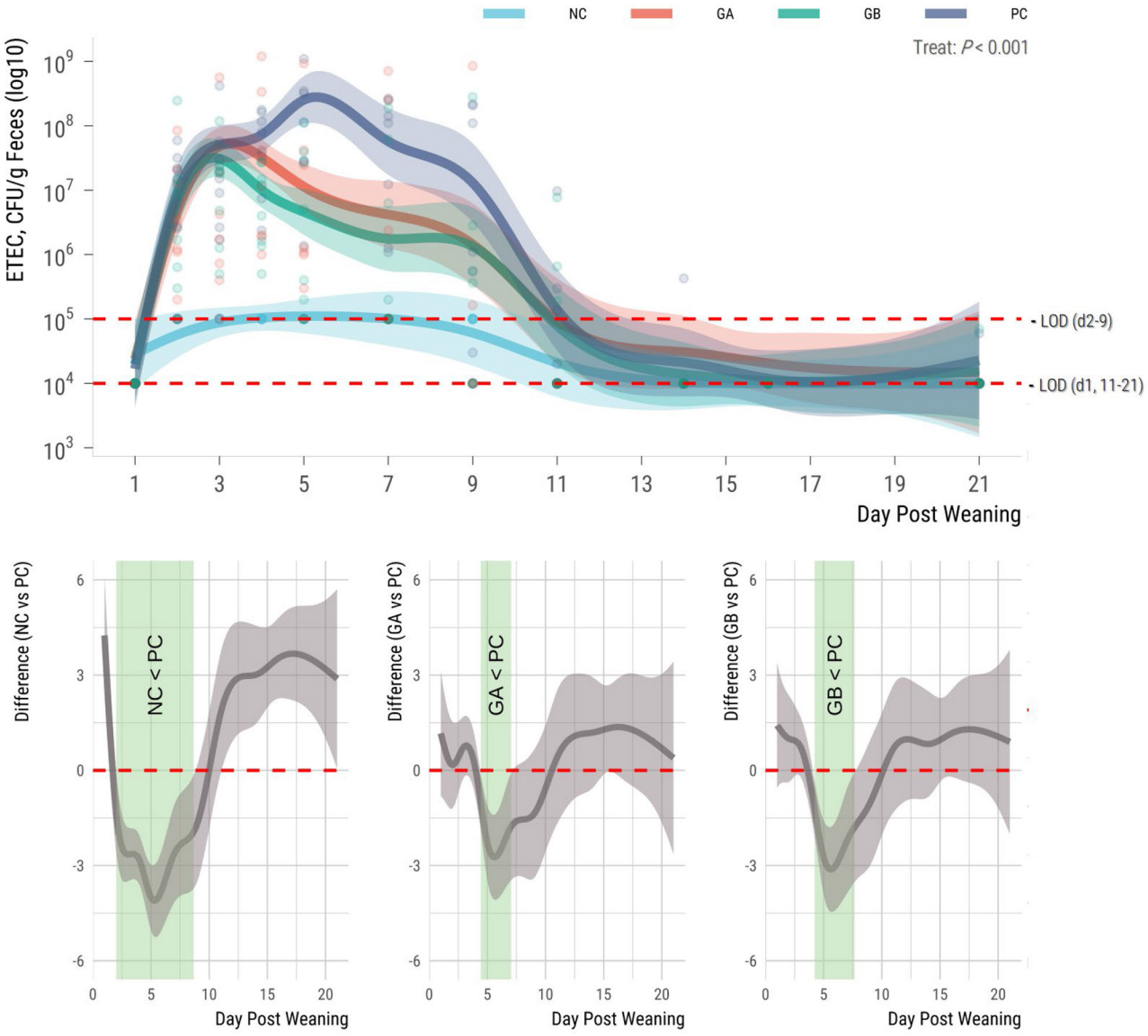

FIGURE 3
Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and dietary supplementation with plant combinations on fecal shedding of ETEC F18 [plate counts; Colony forming units (CFU)] in organically reared weaner pigs. **(Top panel)** Lines represent trends for each group; shaded regions are 95% confidence intervals. The ETEC F18 was administered orally on days 1 and 2. **(Bottom panel)** Pairwise comparisons against PC; significant differences exist where the 95% empirical Bayesian simultaneous confidence interval does not cover 0 (green shade). NC, non-challenge, standard diet ($n = 8$); PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).

the GA pigs had a higher proportion of monocytes than the NC pigs. On day 5, the GB group had a lower proportion of monocytes than the PC group. On day 21, the PC and GA groups had a lower proportion of lymphocytes than the NC group.

3.5. Plasma acute phase proteins

The acute phase protein concentrations in plasma were influenced by the treatment and day (Table 5). The treatment effect on plasma pigMAP concentration was dependent on the day ($P = 0.002$). On day 7, the pigMAP concentration was lower in the NC and GA groups than in the PC group, whereas the GB group did not differ from the PC group. On day 21, the pigMAP concentration was higher in the GA, GB, and PC than in the NC group. The treatment effect on plasma CRP concentration was dependent on the day ($P < 0.001$). On the 1st day of the study, the GB group had lower levels of CRP compared to the other groups. On days 3 and 5, the GB pigs had a higher CRP concentration than the NC pigs. By the end of the study on day 21, the CRP concentration in the GA and GB pigs was higher than in the NC and PC pigs. The treatment effect on plasma haptoglobin concentration was dependent on the day ($P < 0.001$). On day 5, the haptoglobin concentration in the NC, GA and GB pigs did not differ from the PC group but it was

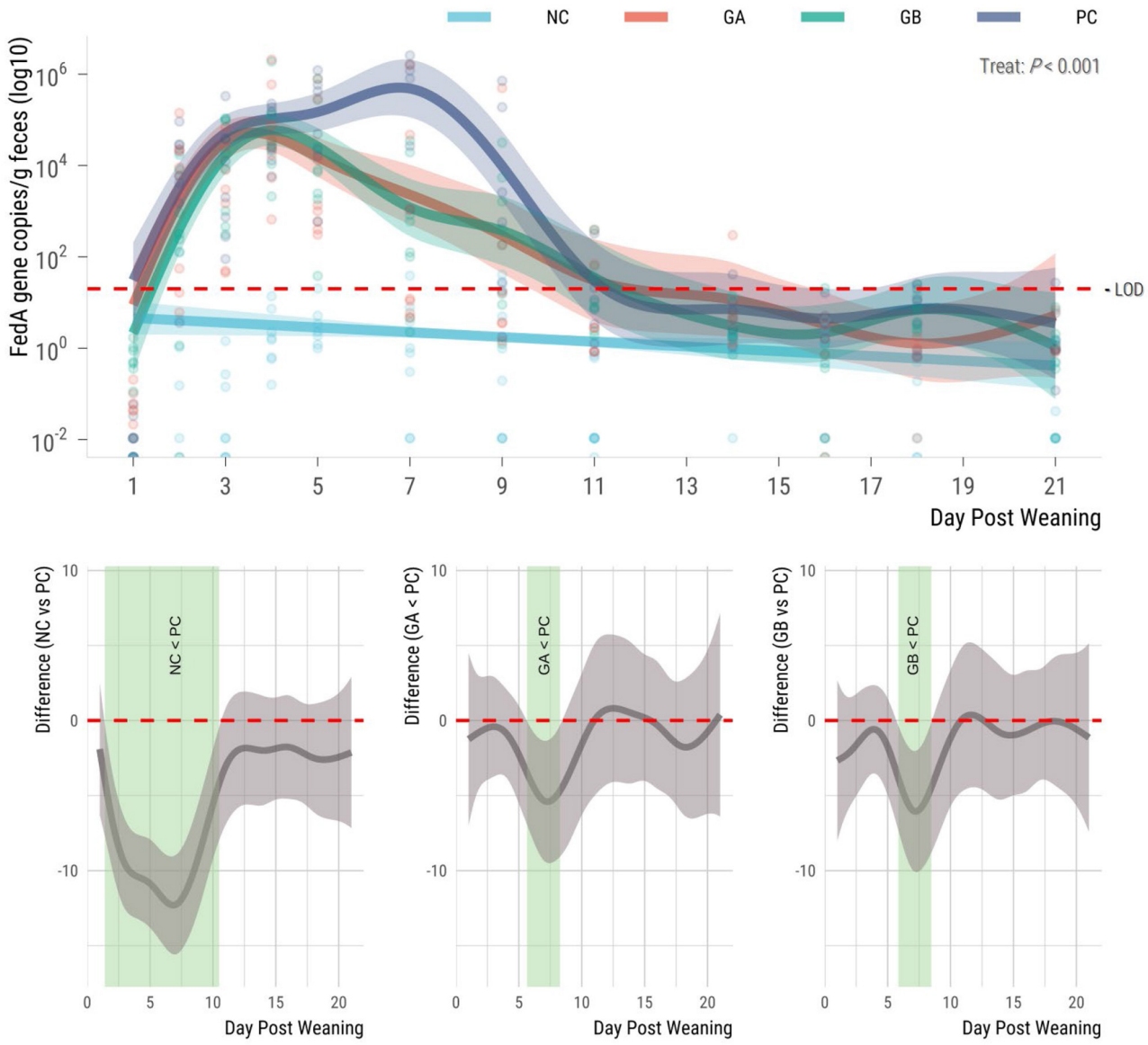

FIGURE 4
Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and dietary supplementation with plant combinations on fecal shedding of the *fedA* gene encoding the ETEC F18 fimbriae type (gene copies/g feces) in organically reared weaner pigs. **(Top panel)** Lines represent trends for each group, shaded regions are 95% confidence intervals. The ETEC F18 was administered orally on days 1 and 2. **(Bottom panel)** Pairwise comparisons against PC; significant differences exist where the 95% empirical Bayesian simultaneous confidence interval does not cover 0 (green shade). NC, non-challenge, standard diet ($n = 8$); PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).

higher in the GB than in the NC pigs. On day 7, the haptoglobin concentration in the NC and GB pigs did not differ from the PC group, whereas that of the GA group was lower than the PC and GB groups. By the end of the study on day 21, the haptoglobin concentration of the NC, GA and GB pigs was lower than the PC pigs.

3.6. 16S rRNA gene sequencing

A total of 147 fecal samples were sequenced. An average of 1,047 ASVs were identified per sample coming from 9,572,592 reads. On average, 78% of the identified ASVs were retained following size filtering, corresponding to 98.5% of the high-quality reads (Supplementary Table 3).

3.6.1. Alpha-diversity

There was no effect of treatment on the observed α-diversity (Figure 6), whereas the treatment effect on Shannon ($P = 0.002$) and Inverse Simpson ($P < 0.001$) indices were dependent on the day. Observed α-diversity generally increased from days 1 to 7 and then decreased again on day 21. On day 7, the GA, GB, and NC pigs

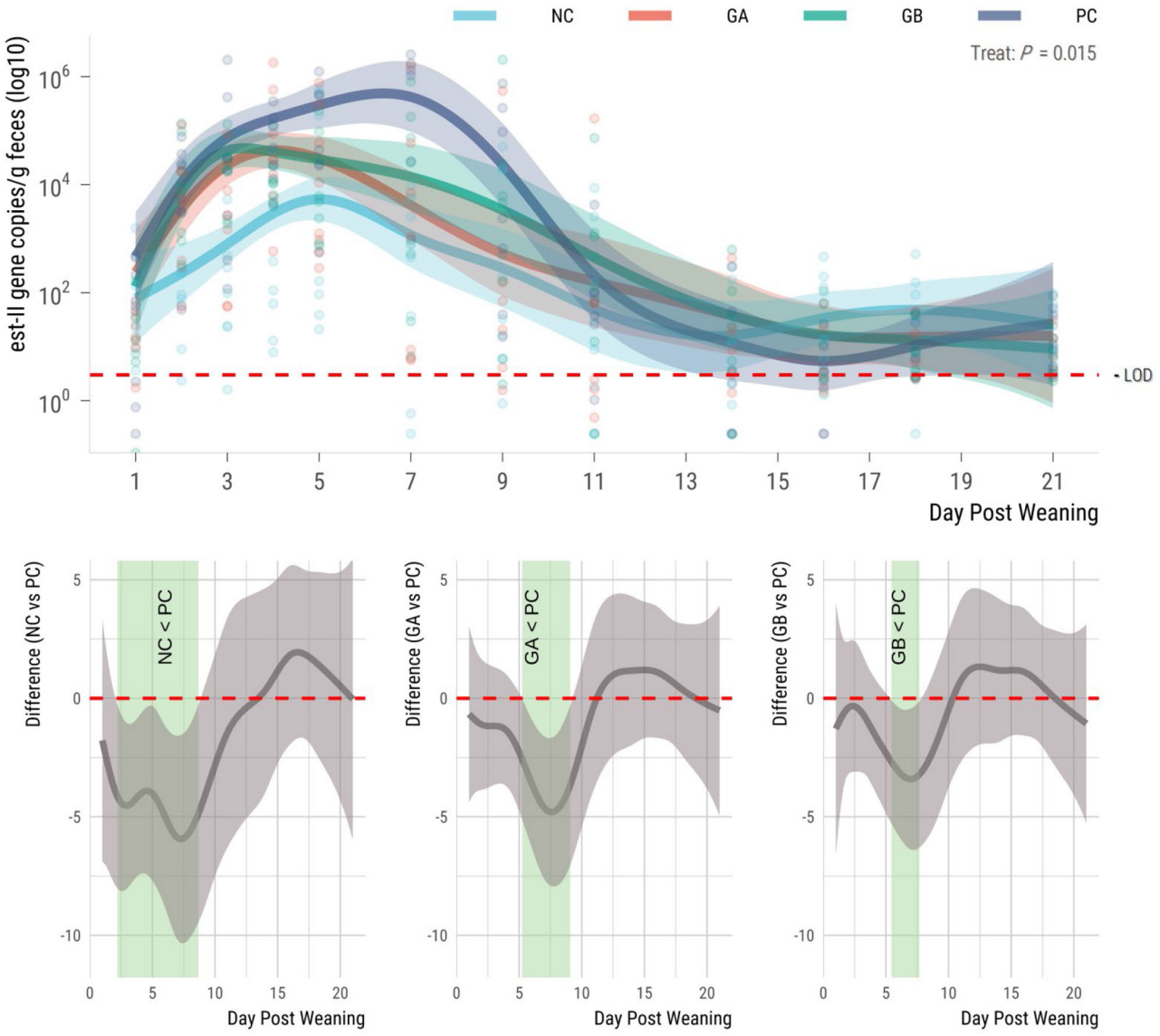

FIGURE 5
Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and dietary supplementation with plant combinations on fecal shedding the *est-II* gene encoding the heat-stabile toxin Stb (gene copies/g feces). **(Top panel)** Lines represent trends for each group, shaded regions are 95% confidence intervals The ETEC F18 was administered orally on days 1 and 2. **(Bottom panel)** Pairwise comparisons against PC; significant differences exist where the 95% empirical Bayesian simultaneous confidence interval does not cover 0 (green shade). NC, non-challenge, standard diet ($n = 8$); PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 +3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).

had a greater Shannon and inverse Simpson α-diversity index than the PC pigs.

3.6.2. Beta-diversity

The effect of treatment on the volatility (distance from the previous observation of individual community structures, i.e., indicator of stability over time) of the fecal microbiota ($P = 0.009$) was dependent on the day (Figure 7). The PC group had a greater microbiota volatility than all other groups on days 7 and 14. No significant difference in the distance from the previous day was observed on days 3 or 21.

Treatments influenced β-diversity of the fecal microbiota (Supplementary Figure 1). On day 1, the GA pigs resembled the NC pigs, while the rest differed. On day 3, the β-diversity of the PC group differed from that of NC, whereas that of the GA and GB groups were similar to that of the NC pigs. On day 7, all groups differed in terms of β-diversity, but the GA and GB groups appeared to cluster closer to the NC piglets. By day 14, the PC group was similar to the NC pigs but differed from the GA and GB groups, and the GA group also differed from the NC. All groups were different by day 21. On days 14 and 21, the GA and GB distances were less dispersed than in the NC and PC groups.

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TABLE 3 Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and dietary supplementation with plant combinations on red blood cell, hemoglobin, hematocrit, and platelet concentrations in organic weaner pigs.

Item[d]	Treatment[e]				SEM[f]	*P*-value		
	NC	PC	GA	GB		Treat	Day	Treat × day
RBC ($10^6/\mu L$)						0.011	0.001	<0.001
Weaning	6.35	6.34	6.13	6.14	0.133			
Day 3	6.20	6.29	6.03	6.14	0.192			
Day 5	6.28	6.53	6.11	6.39	0.197			
Day 7	6.36[a]	7.29[b]	6.32[a]	6.27[a]	0.203			
Day 14	6.52	6.61	5.89	5.97	0.196			
Day 21	6.86[b]	6.75[b]	5.51[a]	5.83[a]	0.197			
HGB (g/L)						0.002	<0.001	<0.001
Weaning	114.02	114.15	114.86	110.11	1.982			
Day 3	111.63	117.37	112.75	112.00	3.781			
Day 5	113.87	118.09	114.04	113.73	2.730			
Day 7	117.63[a]	136.78[b]	121.24[a]	115.37[a]	3.328			
Day 14	123.98[b]	124.80[b]	115.10[a]	112.50[a]	2.701			
Day 21	129.98[b]	127.53[b]	114.93[a]	115.13[a]	2.873			
HCT (%)						0.029	<0.001	<0.001
Weaning	40.38[b]	39.61[ab]	39.92[ab]	37.58[a]	0.702			
Day 3	37.19	38.37	37.90	37.11	1.126			
Day 5	38.78	40.84	38.94	39.72	1.029			
Day 7	38.85[a]	44.44[b]	40.11[a]	38.34[a]	1.132			
Day 14	39.09	39.78	37.21	36.26	1.050			
Day 21	42.04[b]	41.62[b]	37.30[a]	37.10[a]	1.000			
MCV (fL)						0.112	<0.001	<0.001
Weaning	63.64	62.41	64.64	61.25	0.926			
Day 3	60.13	60.99	62.80	60.36	0.906			
Day 5	61.95	62.61	63.74	62.14	1.003			
Day 7	61.32	60.88	63.66	61.11	1.144			
Day 14	60.09	60.28	63.22	60.75	1.153			
Day 21	61.54[a]	61.49[a]	68.05[b]	63.91[ab]	1.453			
MCH (pg)						0.187	<0.001	<0.001
Weaning	17.97	18.03	18.63	17.94	0.254			
Day 3	18.04	18.68	18.70	18.23	0.442			
Day 5	18.19	18.00	18.69	17.80	0.253			
Day 7	18.57	18.71	19.06	18.42	0.291			
Day 14	19.07	18.87	19.46	18.89	0.306			
Day 21	19.01[a]	18.79[a]	20.86[b]	19.84[ab]	0.360			
MCHC (g/L)						0.896	<0.001	0.195
Weaning	282.58	288.09	287.21	292.50	3.701			
Day 3	300.25	305.75	297.88	301.75	3.314			
Day 5	293.87	289.81	292.60	285.18	3.545			
Day 7	303.06	306.49	302.46	301.25	3.715			
Day 14	317.06	313.52	308.26	310.87	3.506			

(Continued)

TABLE 3 (Continued)

Item[d]	Treatment[e]				SEM[f]	*P*-value		
	NC	PC	GA	GB		Treat	Day	Treat × day
Day 21	308.77	305.82	306.59	310.50	3.557			
Reticulocytes (10^9/L)						0.290	<0.001	<0.001
Weaning	209.53[b]	182.13[ab]	171.76[ab]	155.85[a]	13.609			
Day 3	152.65[c]	138.96[bc]	102.60[a]	112.86[ab]	11.647			
Day 5	182.21	153.76	115.46	119.28	18.099			
Day 7	201.78	163.81	166.59	177.35	24.218			
Day 14	172.99[a]	159.15[a]	360.49[b]	227.93[c]	29.300			
Day 21	173.31[a]	183.18[a]	418.74[c]	315.33[b]	19.697			
Platelets (10^9/L)						0.207	0.103	0.706
Weaning	390.78	318.73	376.13	383.34	49.699			
Day 3	345.21	342.52	320.39	319.34	44.776			
Day 5	318.13	359.02	258.40	410.68	47.671			
Day 7	277.25	312.54	343.67	398.50	49.818			
Day 14	354.96	382.32	358.39	405.18	47.179			
Day 21	392.00	380.10	364.25	448.62	47.830			

[abc]Within a row, values that do not share a common superscript differ ($P < 0.05$), Benjamini-Hochberg adjustment.
[d]RBC, Red blood cell count; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean cell volume; MCH, Mean Cell Hemoglobin; MCHC, Mean red blood cell hemoglobin content.
[e]NC, non-challenge ($n = 8$), standard diet; PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 +3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).
[f]Pooled standard error of least squared means.

3.6.3. Relative abundance and differential abundance

Firmicutes (>48%) and Bacteroidetes (>27%) were the most dominant phyla in the feces of all treatment groups throughout the study (Supplementary Figure 2). For most days, the predominant genera were *Prevotella, Lactobacillus, Roseburia,* and *Megasphera* (Figure 8). *Prevotella* was the most abundant (>13%) on most days and in most groups, except for the PC pigs on day 7, where *Escherichia* dominated (19.1%).

Eleven genera were identified as differentially abundant (threshold: $\log_2$FC > 2, $P < 0.05$) in at least one group at a given time when compared against the PC group (Figure 9). The hierarchical dendrogram clustered *Catenibacterium, Dialister, and Mitsoukella* as generally more abundant in the NC, GB, and GA groups than in the PC group, whereas *Lactobacillus, Erysipelonthrix*, and *Campilobacter* clustered as generally less abundant.

On day 7 postweaning, *Gilliamella* was more abundant in NC, GA, and GB than in the PC group. *Escherichia* was less abundant in the NC and GB groups, and *Campylobacter* and *Erysipelothix* were less abundant in NC, GA, and GB than in PC on day 7. Compared to the PC group, *Lactobacillus* was less abundant in the NC group on day 21, in the GA group on days 3 and 7, and in the GB group on day 3. *Catenibacterium* was more abundant in the NC group on day 3, and consistently more abundant in the GA and GB than in the PC pigs. *Dialister* was more abundant in the NC group on days 3 and 7, and in the GA and GB groups on days 3, 7, and 14 than in the PC. *Mitsoukella* was more abundant in the NC, GA, and GB than in the PC pigs on day 3. *Succinivibrio* was less abundant only in the GB pigs on day 21.

Forty-two species were identified as differentially abundant (Supplementary Figure 3). The species that followed a similar behavior to that observed at genus level were: *E. coli, Campylobacter fetus*, *L. johnsonii*, *L. delbrueckii*, *L. pontis*, *L. amylovorus*, *L. reuteri*, and *Catenibacterium mitsoukai*.

4. Discussion

Diarrhea occurrence and bacterial fecal shedding are part of the evaluation of ETEC challenge models for PWD (19). The current study found signs of ETEC infection in the PC pigs (but not in the NC), indicating the validity of the ETEC challenge model. Limited reports exist on ETEC challenge models with organic weaner pigs. In the study of Sørensen et al. (39), pigs were weaned at a similar age as in the current study, and the authors noted difficulties in using the ETEC challenge model to consistently introduce a diarrhea-like state. Hedemann and Bach Knudsen (40) observed low F-DM during 2 days with peaks on days 4 and 5 postweaning (1–2 days postinfection) with pigs weaned at 7 weeks following an ETEC F4 challenge. Our study was able to induce a diarrhea-like state in the PC pigs for ≈5 days consistently differing from the NC group. This was despite the relatively high weaning age and weight of the animals, both of which have been reported as factors that could affect the efficacy of the challenge model (19). The challenge dosage in the current study was higher than that of the two aforementioned studies (10^9 vs. 10^8 CFU/day), which could

TABLE 4 Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and dietary supplementation with plant combinations on white blood cell differential of organic weaner pigs.

Item[d]	Treatment[e]				SEM[f]	*P*-value		
	NC	PC	GA	GB		Treat	Day	Treat × day
WBC (10^9/L)						0.702	0.185	0.419
Weaning	22.95	20.68	19.30	22.82	2.294			
Day 3	22.65	21.89	23.15	26.36	2.082			
Day 5	21.07	23.06	21.48	22.98	2.206			
Day 7	21.54	25.14	24.79	23.19	2.302			
Day 14	21.09	24.74	27.27	22.13	2.187			
Day 21	19.76	22.22	22.93	21.41	2.215			
Neutrophils (%)						0.361	0.035	0.271
Weaning	47.13	41.66	42.49	43.87	2.627			
Day 3	43.38	37.05	35.51	40.19	2.750			
Day 5	42.81	34.33	38.30	41.62	2.796			
Day 7	44.23	40.17	40.66	42.06	2.630			
Day 14	41.30	42.76	41.64	40.61	2.477			
Day 21	37.31	42.14	41.64	38.39	2.507			
Lymphocytes (%)						0.347	<0.001	0.033
Weaning	42.03	49.17	42.91	45.47	3.273			
Day 3	43.74	49.76	49.62	48.30	2.753			
Day 5	47.23	56.05	51.34	49.37	2.484			
Day 7	46.87	53.09	50.14	50.79	2.362			
Day 14	52.70	50.88	51.27	52.55	1.819			
Day 21	57.06^{b}	50.91^{a}	50.31^{a}	55.08^{ab}	1.680			
Monocytes (%)						0.012	<0.001	<0.001
Weaning	3.62^{b}	1.13^{a}	5.72^{c}	4.87^{bc}	0.642			
Day 3	4.61^{a}	5.76^{ab}	6.69^{b}	4.66^{a}	0.497			
Day 5	5.12^{ab}	6.32^{b}	5.90^{ab}	4.54^{a}	0.465			
Day 7	5.47	4.61	5.65	4.21	0.621			
Day 14	5.24	4.42	5.28	4.71	0.663			
Day 21	4.68	5.57	6.17	4.92	0.588			
Eosinophils (%)						0.767	<0.001	0.021
Weaning	7.57	10.04	8.90	6.14	1.217			
Day 3	8.27	7.36	8.16	6.81	1.170			
Day 5	4.81	3.36	4.36	4.58	0.708			
Day 7	3.30	1.92	3.65	2.91	0.569			
Day 14	1.07	1.87	1.63	2.07	0.479			
Day 21	1.09	2.00	1.83	1.61	0.483			
Basophils (%)						1.000	0.905	0.964
Weaning	0.05	0.03	0.10	0.04	0.312			
Day 3	0.00	0.06	0.01	0.03	0.156			
Day 5	0.02	0.03	0.01	0.03	0.194			
Day 7	0.01	0.01	0.00	0.02	0.113			
Day 14	0.00	0.01	0.07	0.05	0.203			
Day 21	0.03	0.03	0.05	0.00	0.182			

[abc]Within a row, values with different superscripts differ ($P < 0.05$), Benjamini-Hochberg adjustment.

[abc]Within a row, values that do not share a common superscript differ ($P < 0.05$), Benjamini-Hochberg adjustment.

[d]WBC, White blood cell count.

[e]NC, non-challenge ($n = 8$), standard diet; PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).

[f]Pooled standard error of least squared means.

TABLE 5 Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and dietary supplementation with plant combinations on pig major acute protein (pigMAP), C-reactive protein (CRP), and haptoglobin (HPT) levels in plasma of organic weaner pigs.

Item	Treatment[d]				SEM[e]	*P*-value		
	NC	PC	GA	GB		Treat	Day	Treat × day
pigMAP (mg/L)						0.151	<0.001	0.002
Weaning	751.96	759.17	786.98	739.83	63.882			
Day 3	733.24	774.91	792.57	790.87	60.513			
Day 5	669.56	805.78	876.11	846.53	71.337			
Day 7	780.51^{a}	1055.81^{b}	770.71^{a}	852.91^{ab}	63.282			
Day 14	634.03	777.25	677.80	711.84	91.095			
Day 21	353.63^{a}	469.95^{b}	522.48^{b}	504.33^{b}	32.603			
CRP (mg/L)						0.044	<0.001	<0.001
Weaning	19.70^{b}	19.16^{b}	21.45^{b}	11.44^{a}	3.632			
Day 3	15.18^{a}	22.47^{ab}	17.51^{ab}	26.51^{b}	4.565			
Day 5	16.16^{a}	22.51^{ab}	19.65^{ab}	29.63^{b}	4.711			
Day 7	24.41	19.86	16.08	28.01	5.455			
Day 14	12.65	8.59	19.10	20.13	4.444			
Day 21	4.16^{a}	6.91^{a}	17.88^{b}	13.34^{b}	2.841			
HPT (mg/mL)						0.002	<0.001	<0.001
Weaning	1.26	1.14	1.22	1.12	0.102			
Day 3	1.65	1.92	1.92	2.00	0.165			
Day 5	1.89^{a}	2.49^{ab}	2.12^{ab}	2.71^{b}	0.214			
Day 7	2.26^{ab}	2.85^{b}	1.83^{a}	2.80^{b}	0.271			
Day 14	1.54	1.66	1.41	2.19	0.242			
Day 21	0.86^{b}	1.60^{c}	0.26^{a}	0.55^{b}	0.165			

[abc]Within a row, values that do not share a common superscript differ ($P < 0.05$), Benjamini-Hochberg adjustment.
[d]NC, non-challenge (n = 8), standard diet; PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).
[e]Pooled standard error of least squared means.

have been a factor determining the effectiveness of the challenge model in the present study.

All pigs started the study in good health. On arrival, ETEC F18 and the virulence gene *fedA* (encoding for the F18 fimbriae) were not detectable, whereas pigs shed detectable levels of the *est-II* gene encoding for the STb toxin. Similar observations have been reported in previous ETEC challenge studies. Spitzer et al. (41) reported the presence of the *est-II* gene but not the F4 encoding gene in the feces of healthy weaned pigs prior to ETEC F4 challenge. Rhouma et al. (42) similarly found genes encoding STa and STb (but not F18 or F4) in the feces of piglets before oral ETEC F4 challenge. Since the piglets did not have diarrhea on arrival, the findings indicate that the fecal presence of *E. coli* positive for toxins but negative for F4 or F18 fimbriae may not always be associated with diarrheal symptoms.

During the 1st week postweaning, the PC pigs displayed increased fecal shedding of ETEC F18 and virulence factor genes, higher fecal scores (more liquid feces), lower F-DM, and decreased growth metrics, which coincide with typical signs of PWD in infection models (19, 43). Furthermore, on day 7 postweaning, the RBC, HGB, and HCT levels were elevated in the PC pigs, with HGB and HCT levels exceeding reference intervals for their age (44, 45). The elevated levels of RBC, HGB, and HCT (on day 7) in the PC pigs could be linked to dehydration, which corresponds to the diarrheal symptoms (2) observed during the 1st week after weaning (5–6 days postinfection).

We observed increased levels of reticulocytes (immature erythrocytes) near the end of the study, a relatively lower RBC, HGB, and HCT, higher MCV, and no change in MCHC in the GA and GB pigs compared to the PC and NC pigs. This is noteworthy because garlic constituents can cause oxidative hemolysis (46) and excessive intake can result in anemia (47). In the current study, although the RBC were slightly below reference levels, the HGB, HCT, MCV, and MCHC were not abnormal, indicating that the pigs were not anemic. This state, in which garlic induces mild chronic hemolysis without resulting in anemia, has been proposed as a potential mechanism involved in the antiplatelet, pro-circulatory, anti-inflammatory, and antiapoptotic effects associated with the therapeutic use of garlic products (48). According to Akgül et al. (48), the health benefits of garlic consumption could be a manifestation of achieving homeostasis, partly mediated by younger RBC. However, because the study was conducted in mice

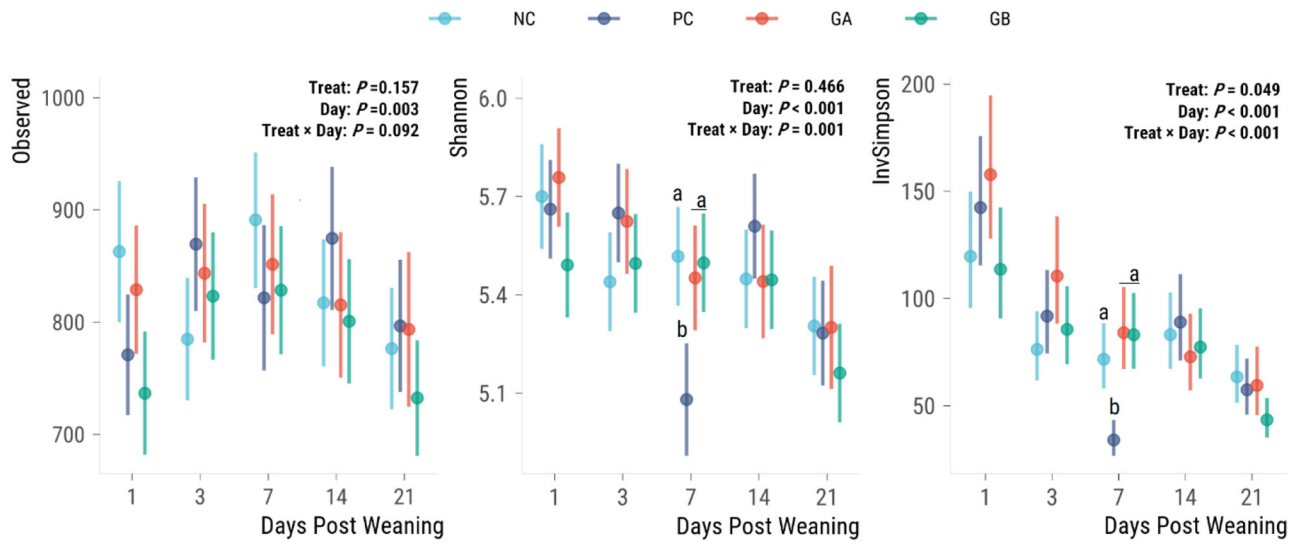

FIGURE 6
Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and supplementation with plant combinations on α-diversity measures of the fecal microbiota. Dots are least squared means and lines are 95% confidence intervals. The ETEC F18 was administered orally on days 1 and 2. [a,b]Values that do not share a common superscript differ ($P < 0.05$), Benjamini-Hochberg adjustment. NC, non-challenge, standard diet ($n = 8$); PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).

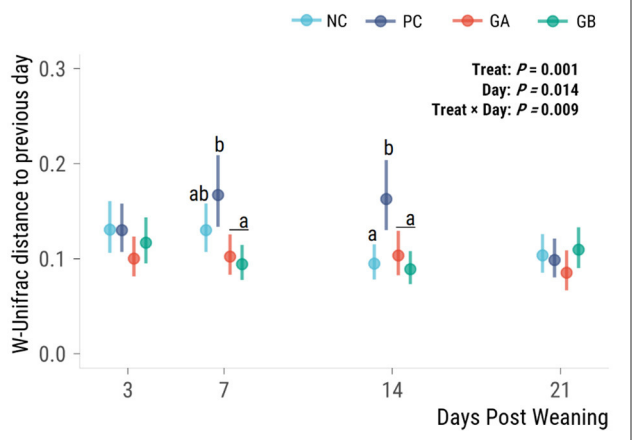

FIGURE 7
Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and supplementation with plant combinations on β-diversity measures of the fecal microbiota. Weighted Unifrac distance difference from the prior time point. Dots are least squared means and lines are 95% confidence intervals. The ETEC F18 was administered orally on days 1 and 2. [a,b]Values that do not share a common superscript differ ($P < 0.05$), Benjamini-Hochberg adjustment. NC, non-challenge, standard diet ($n = 8$); PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).

(48) and there are no follow-up studies, more research on these mechanisms and their relevance for other species is required.

Different types of pathologies and inflammation processes can result in variable kinetic patterns for pigMAP, CRP, haptoglobin, and other acute phase proteins. Moreover, monocytes and particularly neutrophils are expected to play an effector role in response to bacterial infections. The pigMAP, haptoglobin, and monocyte levels followed a similar pattern in all groups in the current study, rising during the 1st week, peaking on days 5 or 7, and then declining (except for monocytes in GB pigs). However, no significant response was observed in the other white blood cell pools, and the concentration of monocytes and neutrophils were within normal limits (44, 45). The weaning process has been shown to cause transient inflammation in piglets that upregulates gut pro-inflammatory cytokines (49). Similarly, elevated levels of pigMAP and haptoglobin have been observed in ETEC-challenged pigs (50, 51). Hence both, weaning-related stress and ETEC challenge, could have contributed to the acute phase response observed under the current experimental conditions. Clapperton et al. (52) documented the positive association between pigMAP, haptoglobin, and monocyte concentrations, which we also observed in the current study. This might be due to activated monocytes producing pro-inflammatory cytokines, particularly interleukin 6, which stimulates hepatic production of pigMAP and haptoglobin.

On days when the PC pigs had acute PWD symptoms (days 5–9 postweaning), the GA and GB pigs had lower fecal scores (more solid feces), higher fecal dry matter, and lower fecal shedding of ETEC F18 bacteria (and associated virulence factors), indicating that GA and GB supplementation provided protection against ETEC-induced diarrhea. It is worth noting that, when compared to the PC group, the pigs in the GA and GB groups shed comparable amounts of ETEC and virulence factors during the first 3 days postinfection, indicating that the GA and GB piglets were infected with ETEC but did not develop PWD. There are no previous reports on the use of GA or GB supplementation in pigs. Dietary supplementation with garlic has been of interest and there are reports in the literature showing increased growth performance (although not always consistent), lower diarrhea incidence, and

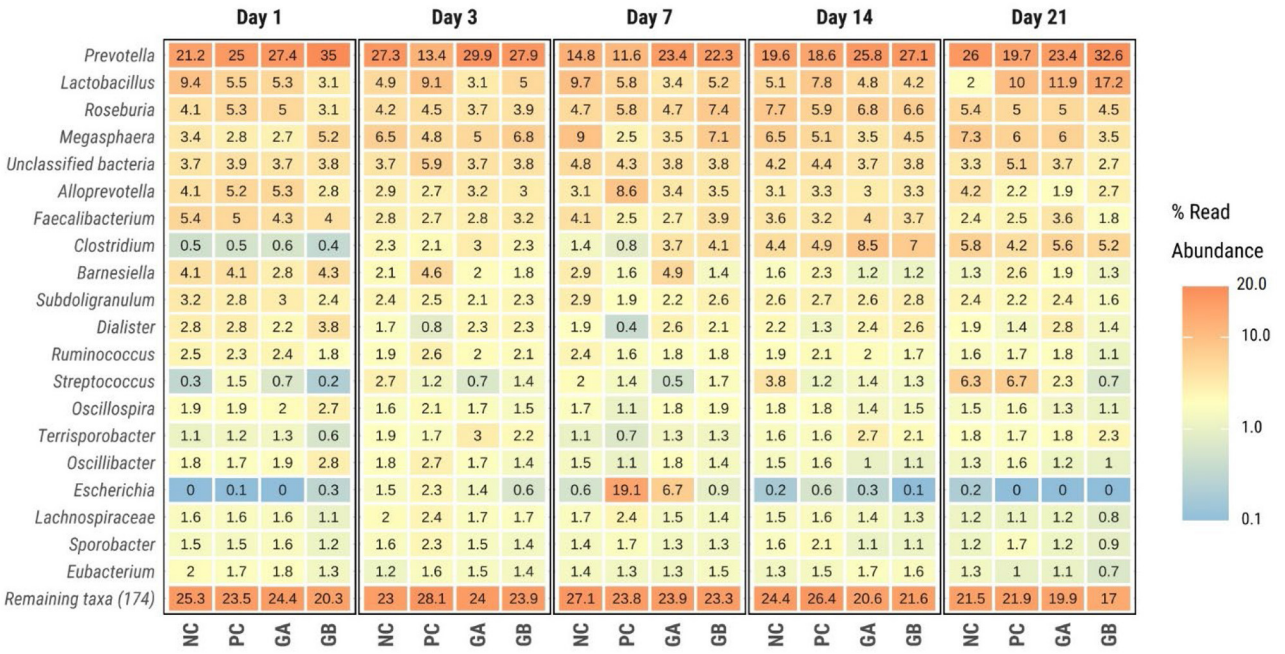

FIGURE 8
Relative abundance of bacterial genera within the feces of pigs challenged with enterotoxigenic *E. coli* (ETEC) F18 after weaning and supplemented with plant combinations. The ETEC F18 was administered orally on days 1 and 2. Values indicate mean relative abundance in percentage of the 20 dominant genera (Y-axis) across treatments (X-axis) and days postweaning (panel). NC, non-challenge, standard diet ($n = 8$); PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).

reduced *E. coli* shedding (53). However, there are few studies evaluating the effectiveness of garlic supplementation under controlled ETEC infection settings. Liu et al. (54) reported reduced serum haptoglobin concentrations, lower fecal scores and less diarrhea frequency in ETEC-challenged pigs fed a diet containing 10 ppm of garlic botanical (extract standardized to contain 40% propyl thiosulfonates), but no change was observed in ETEC fecal shedding. We similarly observed reduced diarrheal symptoms in pigs but accompanied by a reduced ETEC shedding. This point to the antibacterial effect against ETEC of the supplementation used in the current study, in contrast with Liu et al. (54) where antidiarrheal properties were attributed to immunomodulation.

The antibacterial effect observed here may be due to the higher inclusion of garlic, the preparation of the powder (11) or the synergistic action of garlic combined with the acidifying properties of apple pomace and blackcurrant. Allicin from garlic inhibits the growth of *E. coli* by inducing thiol stress and decreasing S-allylmercapto modification of proteins and glutathione pool, resulting in a total reduction in sulfhydryl level (55). The garlic cultivar and the processing method of the garlic powder can affect the potential content of allicin. Therador cultivar was identified as a high-allicin cultivar among over 10 garlic cultivars tested (Jensen, M., personal communication), and the preparation of the powder warranted allicin formation. The acidifying properties of apple pomace and blackcurrant may also prevent pathogen overgrowth because some pathogenic bacteria, including *E. coli*, are susceptible to low pH exposure (7). Indeed, organic acids have been used in piglet diets as antimicrobial agents and proven effective by providing an unfavorable environment for pathogenic bacteria and/or direct bactericidal action (56). Furthermore, the combination of organic acids with phytochemical compounds may synergistically contribute to productive performance reduced ETEC adherence to intestinal epithelium and overall enhanced intestinal health (57). The efficacy of the plant combinations was tested exclusively against ETEC F18 in this study. However, based on the proposed mode of action and our previous *in vitro* studies testing the antibacterial impact of similar plant materials against ETEC F4 (9), it is plausible that the combinations used in the current study may exhibit *in vivo* bactericidal activity against other strains, e.g., ETEC F4.

Because there are no reports in the literature on the composition of the fecal microbiota of ETEC-challenged organically raised weaned pigs, the current study serves as a reference for future research. We used three α-diversity measures to evaluate the effects of GA and GB supplementation on the richness and evenness of the microbiota of ETEC-challenged piglets. Reduced α-diversity (Shannon and Inverse Simpson indices) of the fecal microbiota of PC pigs were observed on day 7 postweaning. This could be a sign of dysbiosis, which is characterized by decreased microbial diversity (58). A clear decrease in α-diversity following diarrheal events has been observed in human infants (59), and some authors have also observed decreased α-diversity in fecal microbiota of diarrheal piglets (58). However, other studies evaluating ETEC challenge effects on fecal microbiota have failed to observe significant differences in α-diversity (60–62). In agreement with our findings, Rhouma et al. (63) reported reduced α-diversity in the fecal microbiota of pigs challenged with ETEC-F4, which was coincided

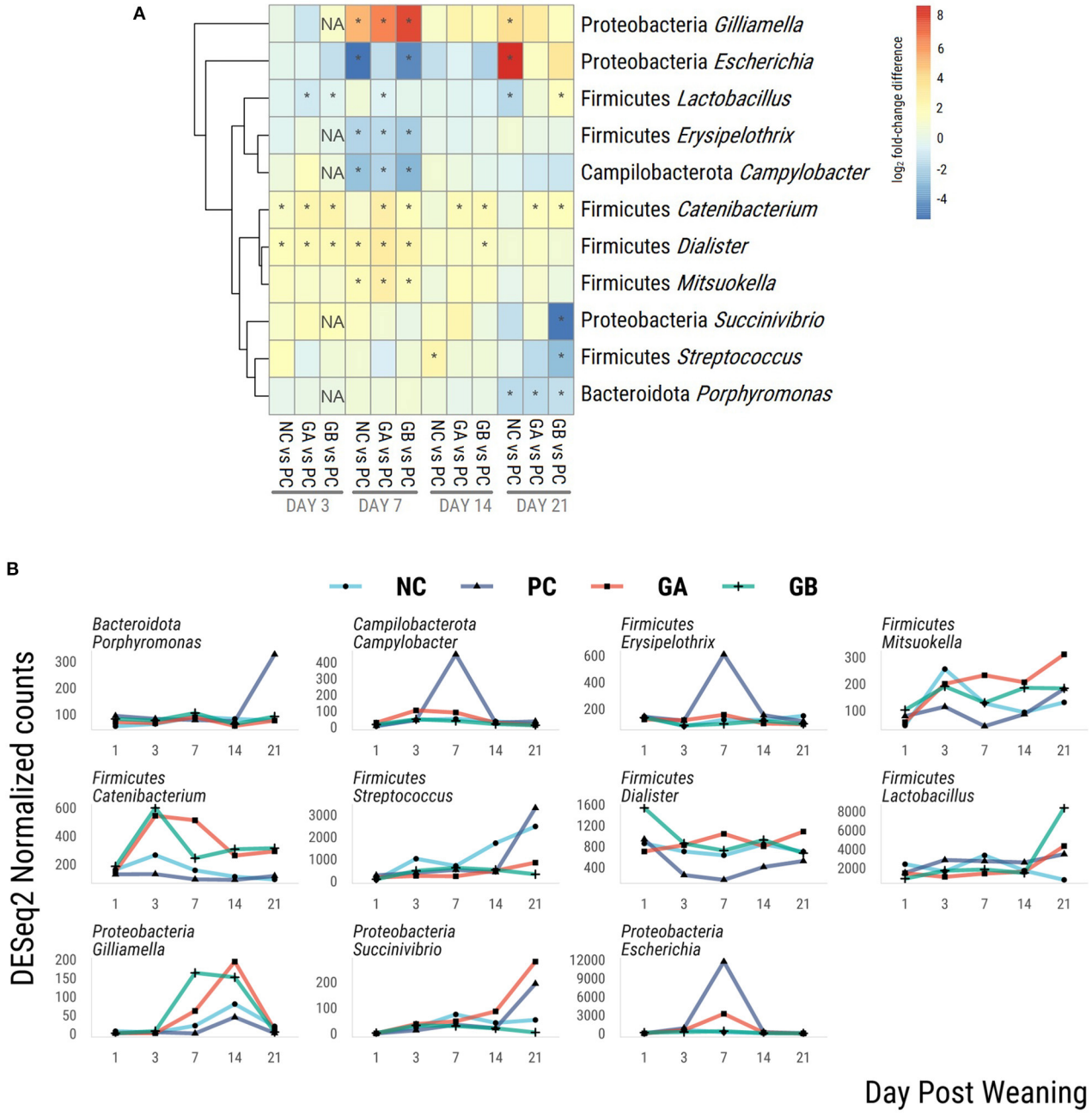

FIGURE 9
Differential abundance of bacterial genera within the feces of pigs challenged with enterotoxigenic *E. coli* (ETEC) F18 after weaning and supplemented with plant combinations. The ETEC F18 was administered orally on days 1 and 2. **(A)** $\log_2$-fold-change difference from PC. *adj-$P <$ 0.05 pairwise comparison against PC within day. NA, *P* value no estimable. **(B)** DESeq2 normalized counts of differentially abundant genera. Only differentially abundant taxa ($\log_2$FC > 2; $P < 0.05$) are represented in the figures. NC, non-challenge, standard diet ($n = 8$); PC, challenged, standard diet (0–7 d, $n = 8$; 7–21 d, $n = 7$); GA, challenged, garlic + apple pomace (3 + 3%; 0–7 d, $n = 8$; 7–21 d, $n = 6$); GB, challenged, garlic + blackcurrant (3 + 3%; $n = 8$).

with acute PWD symptoms, as opposed to other studies that only achieved subclinical states. Similarly, we also observed acute PWD symptoms in the PC group, emulating field conditions of the disease (2). The supplementation of GA and GB prevented a decrease in the richness and evenness of the fecal microbiota of piglets challenged with ETEC. Dietary supplementation with garlic has been reported to increase α-diversity of the fecal microbiota of mice (64) and sows (65). Apple pomace supplementation has also been shown to increase the α-diversity of the cecal mucosa of pigs (66). Interestingly, blackcurrant supplementation showed no effect on α-diversity in broilers (67). A highly diverse microbiota is usually regarded as beneficial to the host, and reduced diversity is associated with acute and chronic diseases (68). The use of antibiotics against ETEC infection often results in reduced diversity of the gut microbiota (58, 63), which in long run may give the opportunity for pathogens to colonize and cause diseases (58).

Thus, the effect of GA or GB supplementation on α-diversity under ETEC challenge conditions can be seen as a positive outcome and is an indication of the targeted nature of the strategy.

We used weighted UniFrac distances to address the degree of differences in fecal microbiota because it considers phylogenetic and abundance information. Additionally, we investigated how the individual microbiota structure changes over time (in relation to the previous observation), known as microbiota volatility (69), as opposed to stability. In this study, we observed increased volatility of the microbiota by day 7 in both ETEC-challenged and unchallenged pigs, and only in the PC group on day 14. This is consistent with previous studies indicating that weaning is associated with abrupt changes in the gut microbiota (70). Furthermore, increased volatility of the gut microbiota has been proposed as a defining feature of (disease-related) dysbiosis (71). Increased volatility has also been linked to stress in mice and humans (31). We found that the ETEC F18 challenge delayed the stabilization of the fecal microbiota after weaning, as observed by the increased volatility; however, this was prevented with GA or GB supplementation.

Throughout the study, the ETEC F18 challenge influenced the microbial community structure (β-diversity) of pigs. Although PWD signs had disappeared in the PC group 2 weeks after weaning, the fecal microbiota composition remained different from that of the NC pigs. Rhouma et al. (63) observed a significant effect of ETEC challenge on β-diversity, even up to 5 weeks after weaning, indicating that the PWD episodes had a long-term impact on piglet fecal microbiota. In the current study, following the ETEC F18 challenge, the microbiota configuration of pigs in the GA and GB groups differed from the PC pigs consistently. Similar effects have been observed 40 days postweaning in pigs supplemented with pharmacological levels of zinc oxide during the nursery period (72), and 21 days postweaning following chito-oligosaccharide supplementation to piglets during an ETEC challenge (73). We only followed the pigs up to 3 weeks after weaning, but considering the aforementioned studies, a lasting effect of GA and GB supplementation on the microbiota structure of pigs may be expected.

During this study, the most abundant phyla within the fecal microbiota were Firmicutes and Bacteroidetes, which agrees with previous reports in weaned piglets (70). The ETEC challenge, as expected, increased the relative abundance of Proteobacteria due to an increase in *E. coli*, particularly during the 1st week postweaning. This in turn coincided with the PWD symptoms and fecal ETEC shedding in the PC pigs. The increased abundance of the *Escherichia* also coincided with the increased abundance of *Campylobacter*, *Erysipelothrix*, and lower abundance of *Catenibacterium*, *Dialister*, and *Mitsoukella*. Previous research has shown an increased abundance of *Campylobacter* after weaning, which is regarded as an opportunistic pathogen and is thought to be linked to the occurrence of PWD (74, 75). *Erysipelothrix* have also been shown to cause disease, particularly in older pigs after the decline of maternal antibodies (76). On the other hand, *Dialister* has been shown to be strongly associated with *Prevotella* and some blood parameters (positively with monocytes, platelets and negatively with eosinophils, and red blood cell parameters) (77). Smith et al. (78) also observed decreased abundance of *Dialister* and *Catenibacterium* following ETEC F18 challenge. *Mitsuokella* has been positively associated with body weight, along with *Prevotella* (79). In agreement with our findings, Duarte et al. (80) also reported a reduced abundance of *Mitsuokella* in response to ETEC F18 challenge. *Mitsuokella* has been shown to be more abundant in pigs fed diets supplemented with bacitracin and challenged with ETEC F18 (81). Thus, the ETEC challenge was associated with an increase in the abundance of potentially pathogenic bacteria at the expense of commensal bacteria.

Supplementation with GB prevented the overgrowth of *E. coli* after challenge, whereas the difference between GA and PC pigs was not significant. *Catenibacterium mitsuokai* was consistently more abundant in the GA and GB groups than in the PC. This genus is a Gram-positive anaerobe that produces acetic, lactic, butyric and iso-butyric acids from glucose (82). *Catenibacterium* has previously been reported as exclusively present in pigs supplemented with inulin, when compared to others receiving different dietary fiber types (83) and when supplementing with probiotics (84). Others have also shown that apple pomace supplementation increases the abundance of *Catenibacterium* in piglets (66). However, there is no reported evidence linking garlic or blackcurrant supplementation to *Catenibacterium*. This suggests that *Catenibacterium* abundance may be related to the dietary fiber or polyphenols in GA and GB diets. We observed an increased abundance of several species of *Lactobacillus* in the GB group toward the end of the study. *Lactobacillus* is well-known to promote immune cell homeostasis and intestinal health in the host. In agreement with our findings, feeding rats a diet supplemented with blackcurrant extract resulted in a significant increase in the cecal counts of lactobacilli (85). Aged garlic extract supplementation in humans resulted in increased fecal microbial richness, with a significant increase in *Lactobacillus* and *Clostridia* abundance (86). In contrast, Colombino et al. (67) observed reduced abundance of *Lactobacillus* in the excreta of broilers fed blackcurrant pomace. The increased abundance of *Lactobacillus* in our study and others might be related to a prebiotic effect of garlic and blackcurrant. The GB group also had a consistently higher abundance of *Prevotella copri*. *Prevotella* is expected to increase after weaning and to remain more abundant in piglets during the nursery and growing stages (87). The evidence regarding the association between *Prevotella* and diarrhea has been contradictory, but it appears that animals with higher *Prevotella* abundance may have better protection against diarrhea (87). *Prevotella* is widely associated with plant polysaccharide consumption (88), thus comparative advantage for these substrates might have contributed to the higher abundance in the GB group.

The plant inclusion level in the feed was based on expected concentration in the stomach and confirmed *in vitro* bactericidal activity in porcine gastric digesta, with no observed adverse effects on feed intake or toxicity. A higher dose may be more effective, but limitations regarding toxicity, diet formulation, and economics need to be considered. Both the GA and the GB were successful in preventing ETEC (and other potentially pathogenic bacteria) proliferation, thereby preventing PWD development. Furthermore, GA and GB had no adverse effect on piglet fecal microbiota, favoring the abundance of commensal bacteria. Nevertheless, it appeared that GB supplementation was more effective than GA. We observed that the pH of the blackcurrant powder (2.81 ± 0.01) was lower than that of the apple pomace powder (3.28 ± 0.01) and the ABC-4 of the GB diet was lower than that of

the GA diet. Apples harvested before maturity normally have a higher content of organic acids and display lower pH than mature apples and thus unripe apples may be a resource to study more in future. Ascorbic acid and anthocyanins from blackcurrant have shown high stability following simulated gastric digestion (89). The higher acidifying properties of the GB treatment may have therefore contributed to the greater effectiveness observed. However, other mechanisms also seem to play a role, particularly with regard to microbiota modulation. Esposito et al. (90) reported, for example, that blackcurrant anthocyanins have an anti-inflammatory effect that may be mediated by interaction with the local microbiota and their functions in the intestine. The dietary fiber fraction may also have an impact on the gut microbiome, particularly the fermentative species, and thus on the production of lactic acid and short-chain fatty acids, which may further lower the pH locally (57). However, the dietary fiber profiles of the experimental diets used in this study were similar, except for a higher fructan content in GA and GB diets. Thus, more research is required to investigate the roles of dietary fiber and bioactive compounds in GB (e.g., anthocyanins), as well as potential interactions with the gut microbiota.

5. Conclusion

In conclusion, the ETEC F18 challenge as conducted here resulted in decreased growth, evident signs of PWD, and increased abundance of ETEC and other harmful bacteria in the feces of organically raised piglets weaned at 7 weeks of age. The weaning process and the ETEC infection induced high levels of acute phase response parameters during the 1st week postweaning. Dietary GA and GB supplementation offered protection against PWD caused by ETEC F18 infection, inhibiting bacterial pathogen proliferation while maintaining fecal microbiota diversity, reducing volatility of the microbiota structure, and favoring the abundance of beneficial bacteria. The use of GA and GB provides a safe nutritional strategy for management of postweaning diarrhea caused by *E. coli* F18 infection and possibly other ETEC strains. Thereby reducing the need for veterinary medical interventions in organic- and possibly conventional pig production.

Further research using multi-omics approaches may be useful to further investigate the mode of action of GA and GB supplementation and the modulation of gut microbiome, as well as the effects on oxidative capacity and immunological regulation. Further research into the role of GA or GB supplementation in protection against other pathogenic infections is warranted. Furthermore, the long-term effects of postweaning GA or GB supplementation would be relevant to investigate.

Data availability statement

The data presented in the study are deposited in the European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena/browser/home), accession number PRJEB57915.

Ethics statement

The animal study was reviewed and approved by Danish Animal Experiments Inspectorate Ministry of Food, Agriculture and Fisheries Danish Veterinary and Food Administration License 2017-15-0201-01270.

Author contributions

MJ, NC, and OH contributed to conception and design of the study. KJB, NC, and PC curated the data. KJB and PC contributed to the bioinformatic and statistical analyses. MJ, NC, OH, PL, GG, and PC provided scientific input and edited the manuscript. KJB wrote the first draft of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1095160/full#supplementary-material

References

1. Rhouma M, Fairbrother JM, Beaudry F, Letellier A. Postweaning diarrhea in pigs: Risk factors and non-colistin-based control strategies. *Acta Vet Scand.* (2017) 59:31. doi: 10.1186/s13028-017-0299-7

2. Luppi A. Swine enteric colibacillosis: Diagnosis, therapy and antimicrobial resistance. *Porc Health Manag.* (2017) 3:16. doi: 10.1186/s40813-017-0063-4

3. Bonetti A, Tugnoli B, Piva A, Grilli E. Towards zero zinc oxide: Feeding strategies to manage postweaning diarrhea in piglets. *Animals.* (2021) 11:642. doi: 10.3390/ani11030642

4. Nunan C. *Ending Routine Farm Antibiotic Use in Europe. Achieving Responsible Farm Antibiotic Use through Improving Animal Health and Welfare in Pig and Poultry Production. European Public Health Alliance, February 2022. Report No 1.* (2022). Available online at: www.epha.org/ending-routine-farm-antibiotic-use/ (accessed August 20, 2022).

5. Leeb C, Hegelund L, Edwards S, Mejer H, Roepstorff A, Rousing T, et al. Animal health, welfare and production problems in organic weaner pigs. *Org Agric.* (2014) 4:123–33. doi: 10.1007/s13165-013-0054-y

6. Kruse AB, Kristensen CS, Lavlund U, Stege H. Antimicrobial prescription data in Danish national database validated against treatment records in organic pig farms and analysed for associations with lesions found at slaughter. *BMC Vet Res.* (2019) 15:218. doi: 10.1186/s12917-019-1913-x

7. Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. Gastrointestinal health and function in weaned pigs: A review of feeding strategies to control postweaning diarrhoea without using in-feed antimicrobial compounds. *J Anim Physiol Anim Nutr.* (2013) 97:207–37. doi: 10.1111/j.1439-0396.2012.01284.x

8. Ayrle H, Mevissen M, Kaske M, Nathues H, Gruetzner N, Melzig M, et al. Medicinal plants – Prophylactic and therapeutic options for gastrointestinal and respiratory diseases in calves and piglets? A systematic review. *BMC Vet Res.* (2016) 12:89. doi: 10.1186/s12917-016-0714-8

9. Canibe N, Højberg O, Grevsen K, Jensen M. Plants with anitimicrobial effects to combat postweaning diarrhea in piglets—Allicin and acid-containing species. In: *14th Advances in Animal Biosciences: Proceedings of the 14th International Symposium on Digestive Physiology of Pigs (DPP2018).* Brisbane: Cambridge University Press (2018), p. S90–S91. doi: 10.1017/S2040470018000225

10. Putnik P, Gabrić D, Roohinejad S, Barba FJ, Granato D, Mallikarjunan K, et al. An overview of organosulfur compounds from *Allium* spp: From processing and preservation to evaluation of their bioavailability, antimicrobial, and anti-inflammatory properties. *Food Chem.* (2019) 276:680–91. doi: 10.1016/j.foodchem.2018.10.068

11. Lawson LD, Gardner CD. Composition, stability, and bioavailability of garlic products used in a clinical trial. *J Agric Food Chem.* (2005) 53:6254–61. doi: 10.1021/jf050536+

12. Nayak PK, Chandrasekar CM, Sundarsingh A, Kesavan RK. Effect of *in-vitro* digestion on the bio active compounds and biological activities of fruit pomaces. *J Food Sci Technol.* (2020) 57:4707–15. doi: 10.1007/s13197-020-04507-1

13. Ezzat SM, Salama M, El Kersh D, Salem M. Apple pomace as a source of nutraceuticals. In: C Egbuna, B Sawicka, J Khan, editors, *Food and Agricultural Byproducts as Important Source of Valuable Nutraceuticals*. Cham: Springer International Publishing (2022). p. 75–86.

14. Wu L, Sanguansri L, Augustin MA. Protection of epigallocatechin gallate against degradation during *in vitro* digestion using apple pomace as a carrier. *J Agric Food Chem.* (2014) 62:12265–70. doi: 10.1021/jf504659n

15. Gopalan A, Reuben SC, Ahmed S, Darvesh AS, Hohmann J, Bishayee A. The health benefits of blackcurrants. *Food Func.* (2012) 3:795–809. doi: 10.1039/c2fo30058c

16. Puupponen-Pimiä R, Nohynek L, Meier C, Kähkönen M, Heinonen M, Hopia A, et al. Antimicrobial properties of phenolic compounds from berries. *J Appl Microbiol.* (2001) 90:494–507. doi: 10.1046/j.1365-2672.2001.01271.x

17. Landbo A-K, Meyer AS. Effects of different enzymatic maceration treatments on enhancement of anthocyanins and other phenolics in black currant juice. *Innov Food Sci Emerg Technol.* (2004) 5:503–13. doi: 10.1016/j.ifset.2004.08.003

18. Rybak ME, Calvey EM, Harnly JM. Quantitative determination of allicin in garlic: Supercritical fluid extraction and standard addition of alliin. *J Agric Food Chem.* (2004) 52:682–7. doi: 10.1021/jf034853x

19. Luise D, Lauridsen C, Bosi P, Trevisi P. Methodology and application of *Escherichia coli* F4 and F18 encoding infection models in postweaning pigs. *Anim Sci Biotechnol.* (2019) 10:7. doi: 10.1186/s40104-019-0352-7

20. Tybirk P, Sloth N, Kjeldsen N, Shooter L. *Danish Nutrient Requirement Standards (in Danish: Normer for Næringsstoffer)*. Axelborg: SEGES Pig Research Centre (2015).

21. Carstensen L, Ersbøll AK, Jensen KH, Nielsen JP. *Escherichia coli* postweaning diarrhoea occurrence in piglets with monitored exposure to creep feed. *Vet Microbiol.* (2005) 110:113–23. doi: 10.1016/j.vetmic.2005.07.011

22. Lawlor PG, Lynch PB, Caffrey PJ, O'Reilly JJ, O'Connell MK. Measurements of the acid-binding capacity of ingredients used in pig diets. *Ir Vet J.* (2005) 58:447. doi: 10.1186/2046-0481-58-8-447

23. AOAC. Official methods of analysis of AOAC International. *J AOAC Int.* (1997) 80:127A. doi: 10.1093/jaoac/80.6.127A

24. Jensen SK. Improved bligh and dyer extraction procedure. *Lipid Technol.* (2008) 20:280–1. doi: 10.1002/lite.200800074

25. Hansen B. Determination of nitrogen as elementary N, an alternative to Kjeldahl. *Acta Agric Scan.* (1989) 39:113–8. doi: 10.1080/00015128909438504

26. EEC. Commission directive 98/64/Ec of 3 september 1998 establishing community methods of analysis for the determination of amino-acids, crude oils and fats, and olaquindox in feeding stuffs and amending directive 71/393/Eec. *Off J Eur Communities.* (1998) 1998:14–28.

27. Knudsen KEB. Carbohydrate and lignin contents of plant materials used in animal feeding. *Anim Feed Sci Technol.* (1997) 67:319–38. doi: 10.1016/S0377-8401(97)00009-6

28. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. Dada2: High-resolution sample inference from illumina amplicon data. *Nat Methods.* (2016) 13:581–3. doi: 10.1038/nmeth.3869

29. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The silva ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* (2012) 41(D1):D590–D6. doi: 10.1093/nar/gks1219

30. McMurdie PJ, Holmes S. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE.* (2013) 8:e61217. doi: 10.1371/journal.pone.0061217

31. Bastiaanssen TFS, Gururajan A, van de Wouw M, Moloney GM, Ritz NL, Long-Smith CM, et al. Volatility as a concept to understand the impact of stress on the microbiome. *Psychoneuroendocrinology.* (2021) 124:105047. doi: 10.1016/j.psyneuen.2020.105047

32. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-Seq data with Deseq2. *Genome Biol.* (2014) 15:550. doi: 10.1186/s13059-014-0550-8

33. Pedersen EJ, Miller DL, Simpson GL, Ross N. Hierarchical generalized additive models in ecology: An introduction with Mgcv. *PeerJ.* (2019) 7:e6876. doi: 10.7717/peerj.6876

34. Wood SN. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. *J R Stat Soc B.* (2011) 73:3–36. doi: 10.1111/j.1467-9868.2010.00749.x

35. Simpson GL, Singmann H. *Package 'Gratia'. R Package Version 073.* (2022). Available online at: https://CRAN.R-project.org/package=gratia (accessed October 15, 2022).

36. Mundo AI, Tipton JR, Muldoon TJ. Generalized additive models to analyze nonlinear trends in biomedical longitudinal data using R: Beyond repeated measures anova and linear mixed models. *Stat Med.* (2022) 41:4266–83. doi: 10.1002/sim.9505

37. Lüdecke D, Ben-Shachar MS, Patil I, Waggoner P, Makowski D. Performance: An R package for assessment, comparison and testing of statistical models. *J Open Source Softw.* (2021) 6:60. doi: 10.21105/joss.03139

38. Oksanen J. *Vegan: Community Ecology Package. R Package Version 26-4*. (2022). Available online at: https://CRAN.R-project.org/package=vegan (accessed August 5, 2022).

39. Sørensen MT, Vestergaard EM, Jensen SK, Lauridsen C, Højsgaard S. Performance and diarrhoea in piglets following weaning at seven weeks of age: Challenge with *E. coli* O 149 and effect of dietary factors. *Livest Sci.* (2009) 123:314–21. doi: 10.1016/j.livsci.2008.12.001

40. Hedemann MS, Bach Knudsen KE. A Diet containing dried chicory root does not protect against postweaning diarrhoea in an *E. coli* challenge model using piglets weaned at 7 weeks of age. *Livest Sci.* (2010) 133:232–5. doi: 10.1016/j.livsci.2010.06.073

41. Spitzer F, Vahjen W, Pieper R, Martinez-Vallespin B, Zentek J. A standardised challenge model with an enterotoxigenic F4+ *Escherichia coli* strain in piglets assessing clinical traits and faecal shedding of FAE and EST-II toxin genes. *Arch Anim Nutr.* (2014) 68:448–59. doi: 10.1080/1745039X.2014.968701

42. Rhouma M, Fairbrother JM, Thériault W, Beaudry F, Bergeron N, Laurent-Lewandowski S, et al. The fecal presence of enterotoxin and F4 genes as an indicator of efficacy of treatment with colistin sulfate in pigs. *BMC Microbiol.* (2017) 17:6. doi: 10.1186/s12866-016-0915-0

43. Becker SL, Li Q, Burrough ER, Kenne D, Sahin O, Gould SA, et al. Effects of an F18 enterotoxigenic *Escherichia coli* challenge on growth performance, immunological status, and gastrointestinal structure of weaned pigs and the potential protective effect of direct-fed microbial blends. *J Anim Sci.* (2020) 98:skaa113. doi: 10.1093/jas/skaa113

44. Cooper CA, Moraes LE, Murray JD, Owens SD. Hematologic and biochemical reference intervals for specific pathogen free 6-week-old hampshire-yorkshire crossbred pigs. *J Anim Sci Biotechnol.* (2014) 5:5. doi: 10.1186/2049-1891-5-5

45. Ventrella D, Dondi F, Barone F, Serafini F, Elmi A, Giunti M, et al. The biomedical piglet: Establishing reference intervals for haematology and clinical chemistry parameters of two age groups with and without iron supplementation. *BMC Vet Res.* (2017) 13:23. doi: 10.1186/s12917-017-0946-2

46. Munday R. Harmful and beneficial effects of organic monosulfides, disulfides, and polysulfides in animals and humans. *Chem Res Toxicol.* (2012) 25:47–60. doi: 10.1021/tx200373u

47. Saastamoinen M, Särkijärvi S, Hyyppä S. Garlic (*Allium sativum*) supplementation improves respiratory health but has increased risk of lower hematologic values in horses. *Animals.* (2019) 9:ani9010013. doi: 10.3390/ani9010013

48. Akgül B, Lin KW, Ou Yang HM, Chen YH, Lu TH, Chen CH, et al. Garlic accelerates red blood cell turnover and splenic erythropoietic gene expression in mice: Evidence for erythropoietin-independent erythropoiesis. *PLoS ONE.* (2010) 5:e15358. doi: 10.1371/journal.pone.0015358

49. Pié S, Lallés JP, Blazy F, Laffitte J, Séve B, Oswald IP. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. *J Nutr.* (2004) 134:641–7. doi: 10.1093/jn/134.3.641

50. Lee JS, Awji EG, Lee SJ, Tassew DD, Park YB, Park KS, et al. Effect of *Lactobacillus plantarum* Cjlp243 on the growth performance and cytokine response of weaning pigs challenged with enterotoxigenic *Escherichia coli*. *J Anim Sci.* (2012) 90:3709–17. doi: 10.2527/jas.2011-4434

51. He Y, Kim K, Kovanda L, Jinno C, Song M, Chase J, et al. *Bacillus subtilis*: A potential growth promoter in weaned pigs in comparison to carbadox. *J Anim Sci.* (2020) 98:skaa290. doi: 10.1093/jas/skaa290

52. Clapperton M, Bishop SC, Piñeiro M, Campbell FM, Glass EJ. The association between plasma levels of acute phase proteins, haptoglobin, alpha-1 acid glycoprotein (Agp), pig-map, transthyretin and serum amyloid a (SAA) in large white and meishan pigs. *Vet Immunol Immunopathol.* (2007) 119:303–9. doi: 10.1016/j.vetimm.2007.05.008

53. Chen J, Wang F, Yin Y, Ma X. The nutritional applications of garlic (*Allium sativum*) as natural feed additives in animals. *PeerJ.* (2021) 9:e11934. doi: 10.7717/peerj.11934

54. Liu Y, Song M, Che TM, Almeida JA, Lee JJ, Bravo D, et al. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic *Escherichia coli*. *J Anim Sci.* (2013) 91:5294–306. doi: 10.2527/jas.2012-6194

55. Müller A, Eller J, Albrecht F, Prochnow P, Kuhlmann K, Bandow JE, et al. Allicin induces thiol stress in bacteria through S-allylmercapto modification of protein cysteines. *J Biol Chem.* (2016) 291:11477–90. doi: 10.1074/jbc.M115.702308

56. de Lange CFM, Pluske J, Gong J, Nyachoti CM. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. *Livest Sci.* (2010) 134:124–34. doi: 10.1016/j.livsci.2010.06.117

57. Canibe N, Højberg O, Kongsted H, Vodolazska D, Lauridsen C, Nielsen TS, et al. Review on preventive measures to reduce postweaning diarrhoea in piglets. *Animals.* (2022) 12:2585. doi: 10.3390/ani12192585

58. Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E, Blanquet-Diot S. Gut microbiota dysbiosis in postweaning piglets: Understanding the keys to health. *Trends Microbiol.* (2017) 25:851–73. doi: 10.1016/j.tim.2017.05.004

59. Mortensen MS, Hebbelstrup Jensen B, Williams J, Brejnrod AD, O'Brien Andersen L, Röser D, et al. Stability and resilience of the intestinal microbiota in children in daycare—A 12 month cohort study. *BMC Microbiol.* (2018) 18:223. doi: 10.1186/s12866-018-1367-5

60. Pollock J, Hutchings MR, Hutchings KEK, Gally DL, Houdijk JGM. Changes in the ileal, but not fecal, microbiome in response to increased dietary protein level and enterotoxigenic *Escherichia coli* exposure in pigs. *Appl Environ Microbiol.* (2019) 85:e01252–19. doi: 10.1128/AEM.01252-19

61. Li Q, Peng X, Burrough ER, Sahin O, Gould SA, Gabler NK, et al. Dietary soluble and insoluble fiber with or without enzymes altered the intestinal microbiota in weaned pigs challenged with enterotoxigenic *E. coli* F18. *Front Microbiol.* (2020) 11:10. doi: 10.3389/fmicb.2020.01110

62. Duarte ME, Kim SW. Significance of mucosa-associated microbiota and its impacts on intestinal health of pigs challenged with F18^{+} *E. coli*. *Pathogens.* (2022) 11:589. doi: 10.3390/pathogens11050589

63. Rhouma M, Braley C, Thériault W, Thibodeau A, Quessy S, Fravalo P. Evolution of pig fecal microbiota composition and diversity in response to enterotoxigenic *Escherichia coli* infection and colistin treatment in weaned piglets. *Microorganisms.* (2021) 9:1459. doi: 10.3390/microorganisms9071459

64. Chen K, Xie K, Liu Z, Nakasone Y, Sakao K, Hossain MA, et al. Preventive effects and mechanisms of garlic on dyslipidemia and gut microbiome dysbiosis. *Nutrients.* (2019) 11:1225. doi: 10.3390/nu11061225

65. Satora M, Magdziarz M, Rzasa A, Rypuła K, Płoneczka-Janeczko K. Insight into the intestinal microbiome of farrowing sows following the administration of garlic (*Allium sativum*) extract and probiotic bacteria cultures under farming conditions. *BMC Vet Res.* (2020) 16:442. doi: 10.1186/s12917-020-02659-y

66. Dufourny S, Antoine N, Pitchugina E, Delcenserie V, Godbout S, Douny C, et al. Apple pomace and performance, intestinal morphology and microbiota of weaned piglets—A weaning strategy for gut health? *Microorganisms*. (2021) 9:572. doi: 10.3390/microorganisms9030572

67. Colombino E, Ferrocino I, Biasato I, Cocolin LS, Prieto-Botella D, Zduńczyk Z, et al. Dried fruit pomace inclusion in poultry diet: Growth performance, intestinal morphology and physiology. *J Anim Sci Biotechnol.* (2020) 11:63. doi: 10.1186/s40104-020-00464-z

68. Manor O, Dai CL, Kornilov SA, Smith B, Price ND, Lovejoy JC, et al. Health and disease markers correlate with gut microbiome composition across thousands of people. *Nature Commun.* (2020) 11:5206. doi: 10.1038/s41467-020-18871-1

69. Goodrich Julia K, Di Rienzi Sara C, Poole Angela C, Koren O, Walters William A, Caporaso JG, et al. Conducting a microbiome study. *Cell.* (2014) 158:250–62. doi: 10.1016/j.cell.2014.06.037

70. Massacci FR, Berri M, Lemonnier G, Guettier E, Blanc F, Jardet D, et al. Late weaning is associated with increased microbial diversity and *Faecalibacterium prausnitzii* abundance in the fecal microbiota of piglets. *Anim Microbiome.* (2020) 2:2. doi: 10.1186/s42523-020-0020-4

71. Vandeputte D, De Commer L, Tito RY, Kathagen G, Sabino J, Vermeire S, et al. Temporal variability in quantitative human gut microbiome profiles and implications for clinical research. *Nature Commun.* (2021) 12:6740. doi: 10.1038/s41467-021-27098-7

72. da Silva CA, Bentin LAT, Dias CP, Callegari MA, Facina VB, Dias FTF, et al. Impact of zinc oxide, benzoic acid and probiotics on the performance and cecal microbiota of piglets. *Anim Microbiome.* (2021) 3:86. doi: 10.1186/s42523-021-00151-y

73. Yu M, Meng T, He W, Huang H, Liu C, Fu X, et al. Dietary chito-oligosaccharides improve intestinal immunity *via* regulating microbiota and Th17/Treg balance-related immune signaling in piglets challenged by enterotoxigenic *E. coli*. *J Agric Food Chem.* (2021) 69:15195–207. doi: 10.1021/acs.jafc.1c06029

74. Li Y, Guo Y, Wen Z, Jiang X, Ma X, Han X. Weaning stress perturbs gut microbiome and its metabolic profile in piglets. *Sci Rep.* (2018) 8:18068. doi: 10.1038/s41598-018-33649-8

75. Wang X, Howe S, Wei X, Deng F, Tsai T, Chai J, et al. Comprehensive cultivation of the swine gut microbiome reveals high bacterial diversity and guides bacterial isolation in pigs. *mSystems.* (2021) 6:e0047721. doi: 10.1128/mSystems.00477-21

76. Gerber PF, MacLeod A, Opriessnig T. Erysipelothrix rhusiopathiae serotype 15 associated with recurring pig erysipelas outbreaks. *Vet Rec.* (2018) 182:635. doi: 10.1136/vr.104421

77. Estellé J, Mach Casellas N, Ramayo Caldas Y, Levenez F, Lemonnier G, Denis C, et al. The influence of host's genetics on the gut microbiota composition in pigs and its links with immunity traits. In: *10 World Congress of Genetics Applied to Livestock Production.* (2014). Vancouver. Available online at: https://hal.archives-ouvertes.fr/hal-01193952/file/2014_Estelle_WCGALP_1.pdf (accessed October 5, 2022).

78. Smith BN, Hannas M, Orso C, Martins SMMK, Wang M, Donovan SM, et al. Dietary osteopontin-enriched algal protein as nutritional support in weaned pigs infected with F18-fimbriated enterotoxigenic *Escherichia coli*. *J Anim Sci*. (2020) 98:skaa314. doi: 10.1093/jas/skaa314

79. Ramayo-Caldas Y, Mach N, Lepage P, Levenez F, Denis C, Lemonnier G, et al. Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits. *ISME J.* (2016) 10:2973–7. doi: 10.1038/ismej.2016.77

80. Duarte ME, Tyus J, Kim SW. Synbiotic effects of enzyme and probiotics on intestinal health and growth of newly weaned pigs challenged with enterotoxigenic F18^{+} *Escherichia coli*. *Front Vet Sci*. (2020) 7:573. doi: 10.3389/fvets.2020.00573

81. Xu X, Duarte ME, Kim SW. Postbiotic effects of *Lactobacillus fermentate* on intestinal health, mucosa-associated microbiota, and growth efficiency of nursery pigs challenged with F18+ *Escherichia coli*. *J Anim Sci.* (2022) 100:skac210. doi: 10.1093/jas/skac210

82. Kageyama A, Benno Y. *Catenibacterium mitsuokai* gen. nov, sp nov, a gram-positive anaerobic bacterium isolated from human faeces. *Int J Syst Evol Microbiol.* (2000) 50:1595–9. doi: 10.1099/00207713-50-4-1595

83. Yan H, Potu R, Lu H, Vezzoni de Almeida V, Stewart T, Ragland D, et al. Dietary fat content and fiber type modulate hind gut microbial community and metabolic markers in the pig. *PLoS ONE.* (2013) 8:e59581. doi: 10.1371/journal.pone.0059581

84. Pajarillo EAB, Chae JP, Balolong MP, Kim HB, Park C-S, Kang D-K. Effects of probiotic *Enterococcus faecium* Ncimb 11181 administration on swine fecal microbiota diversity and composition using barcoded pyrosequencing. *Anim Feed Sci Technol.* (2015) 201:80–8. doi: 10.1016/j.anifeedsci.2015.01.011

85. Molan A-L, Liu Z, Kruger M. The ability of blackcurrant extracts to positively modulate key markers of gastrointestinal function in rats. *World J Microbiol Biotechnol.* (2010) 26:1735–43. doi: 10.1007/s11274-010-0352-4

86. Ried K, Travica N, Sali A. The effect of kyolic aged garlic extract on gut microbiota, inflammation, and cardiovascular markers in hypertensives: The gargic trial. *Front. Nutr*. (2018) 5:122. doi: 10.3389/fnut.2018.00122

87. Amat S, Lantz H, Munyaka PM, Willing BP. Prevotella in pigs: The positive and negative associations with production and health. *Microorganisms.* (2020) 8:1584. doi: 10.3390/microorganisms8101584

88. Frese SA, Parker K, Calvert CC, Mills DA. Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome.* (2015) 3:28. doi: 10.1186/s40168-015-0091-8

89. Trych U, Buniowska M, Skapska S, Starzonek S, Marszałek K. The Bioaccessibility of antioxidants in black currant puree after high hydrostatic pressure treatment. *Molecules.* (2020) 25:3544. doi: 10.3390/molecules25153544

90. Esposito D, Damsud T, Wilson M, Grace MH, Strauch R, Li X, et al. Black currant anthocyanins attenuate weight gain and improve glucose metabolism in diet-induced obese mice with intact, but not disrupted, gut microbiome. *J Agric Food Chem.* (2015) 63:6172–80. doi: 10.1021/acs.jafc.5b00963

Antibacterial plant combinations prevent postweaning diarrhea in organically raised piglets challenged with enterotoxigenic *Escherichia coli* F18

Supplementary Material

Table S1 Ingredient composition of the experimental diets (as-fed basis, %)

Item, %	Diets[1]		
	NC - PC	GA	GB
Organic wheat	25.75	25.75	25.75
Barley	22.25	22.25	22.25
Organic oats	18.60	12.60	12.60
Garlic powder	0	3.00	3.00
Apple Pulp powder	0	3.00	0
Blackcurrant powder	0	0	3.00
Fishmeal	6.00	6.00	6.00
Organic soy cake	5.70	5.70	5.70
Horse beans	5.00	5.00	5.00
Organic rye	5.00	5.00	5.00
Potato protein	4.40	4.40	4.40
Organic barley	2.80	2.80	2.80
Organic wheat bran	2.00	2.00	2.00
Calcium carbonate	1.15	1.15	1.15
Monocalcium phosphate	0.47	0.47	0.47
Vitamin + Mineral premix[2]	0.40	0.40	0.40
Vitamin E	0.2	0.2	0.2
NaCl	0.28	0.28	0.28

[1] NC: non-challenge, standard diet; PC: challenged, standard diet; GA: challenged, Garlic and Apple pomace supplementation (3%+3%); GB: challenged, garlic and blackcurrant supplementation (3%+3%).
[2] Provided per kg of diet: 173 mg Fe (iron sulfate), 80 mg Cu (copper sulfate), 80 mg Cu (copper sulfate), 46 mg Mn (manganese oxide), 100 mg Zn (Zinc oxide), 0.30 mg I (calcium iodate), 0.30 mg Se (sodium selenite), 5400 UI vitamin A, 1000 IU vitamin D3, 215 IU vitamin E.

Table S2 Quantitative PCR primer details and assay settings

Primer name	Target sequence	Sequence (5'-3')	Conc[1] (mM)	T_A[2] (°C)	Size[3] (bp)
F18 F	*E. coli* F18 fimbriae (*FedA*)	GGAGGTTAAGGCGTCGAATAG	0.3	62	90
F18 R		CCACCTTTCAGTTGAGCAGTA	0.3		
STb F	*E. coli* STb toxin (*estB*)	TGCCTATGCATCTACACAAT	0.3	59.1	113
STb R		CTCCAGCAGTACCATCTCTA	0.3		

[1]Concentration in qPCR reactions
[2]Annealing temperature
[3]Amplicon size

Table S3 DADA2 read tracking summary

Item	Average
Original (ASVs)	1,047.33
Original (read counts)	65,119.67
Low abundance removed ASV	822.92
Low abundance removed read counts	64,161.07
ASVs retained (%)	78.70
Reads retained (%)	98.52

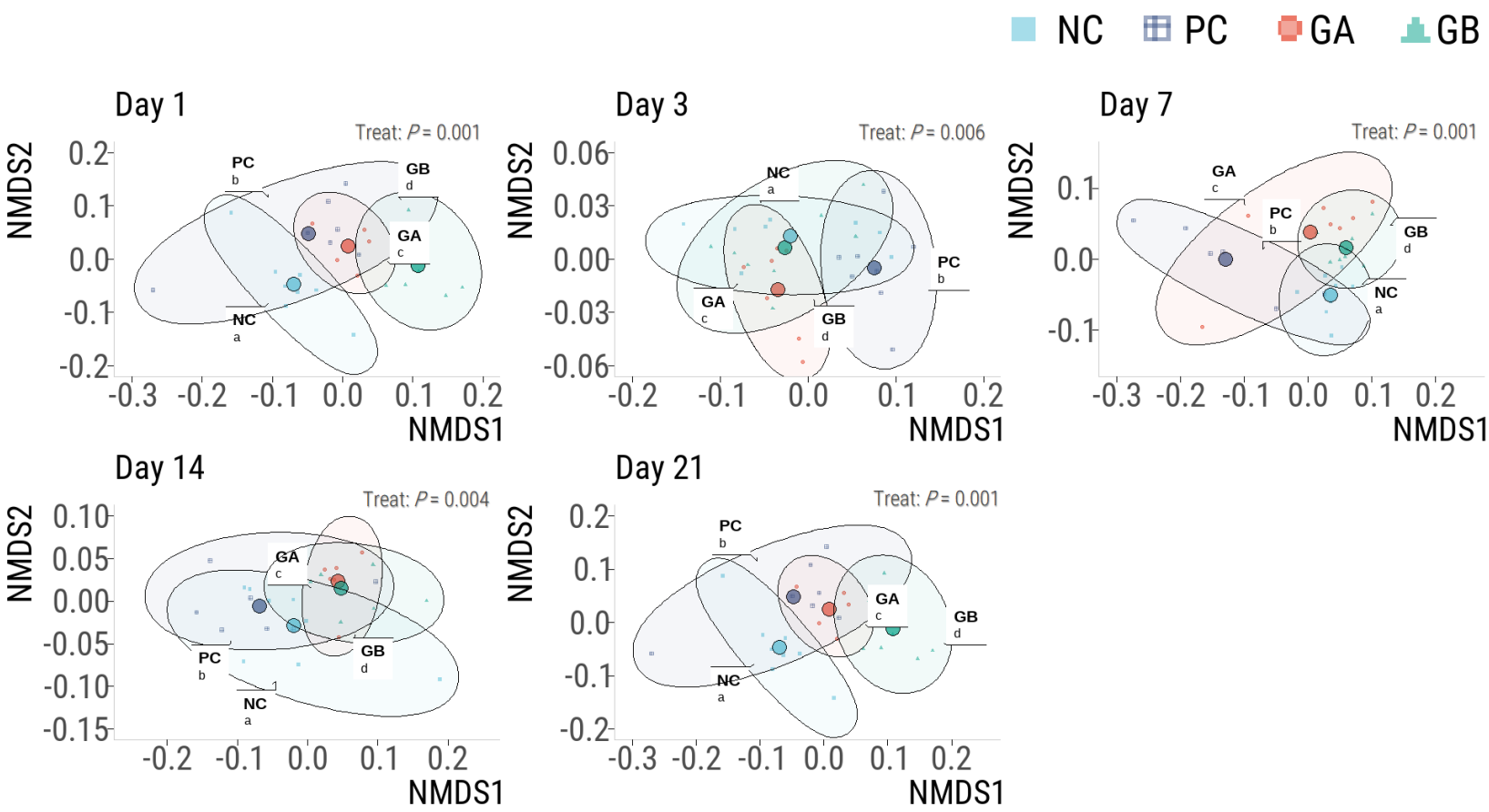

Fig S1 Effect of postweaning enterotoxigenic *E. coli* (ETEC) F18 challenge and supplementation with plant combinations on β-diversity measures of the fecal microbiota. Non-metric multidimensional scaling (NMDS) plot weighted UniFrac dissimilarity distances. Solid dots in ordination are mean centroids. [abcd] Groups that do not share a common superscript differ ($P < 0.05$), Benjamini-Hochberg adjustment. NC: non-challenge, standard diet (n=8); PC: challenged, standard diet (0-7d, n=8; 7-21d, n=7); GA: challenged, Garlic + Apple pomace (3%+3%; 0-7d, n=8; 7-21d, n=6); GB: challenged, Garlic + Blackcurrant (3%+3%; n=8).

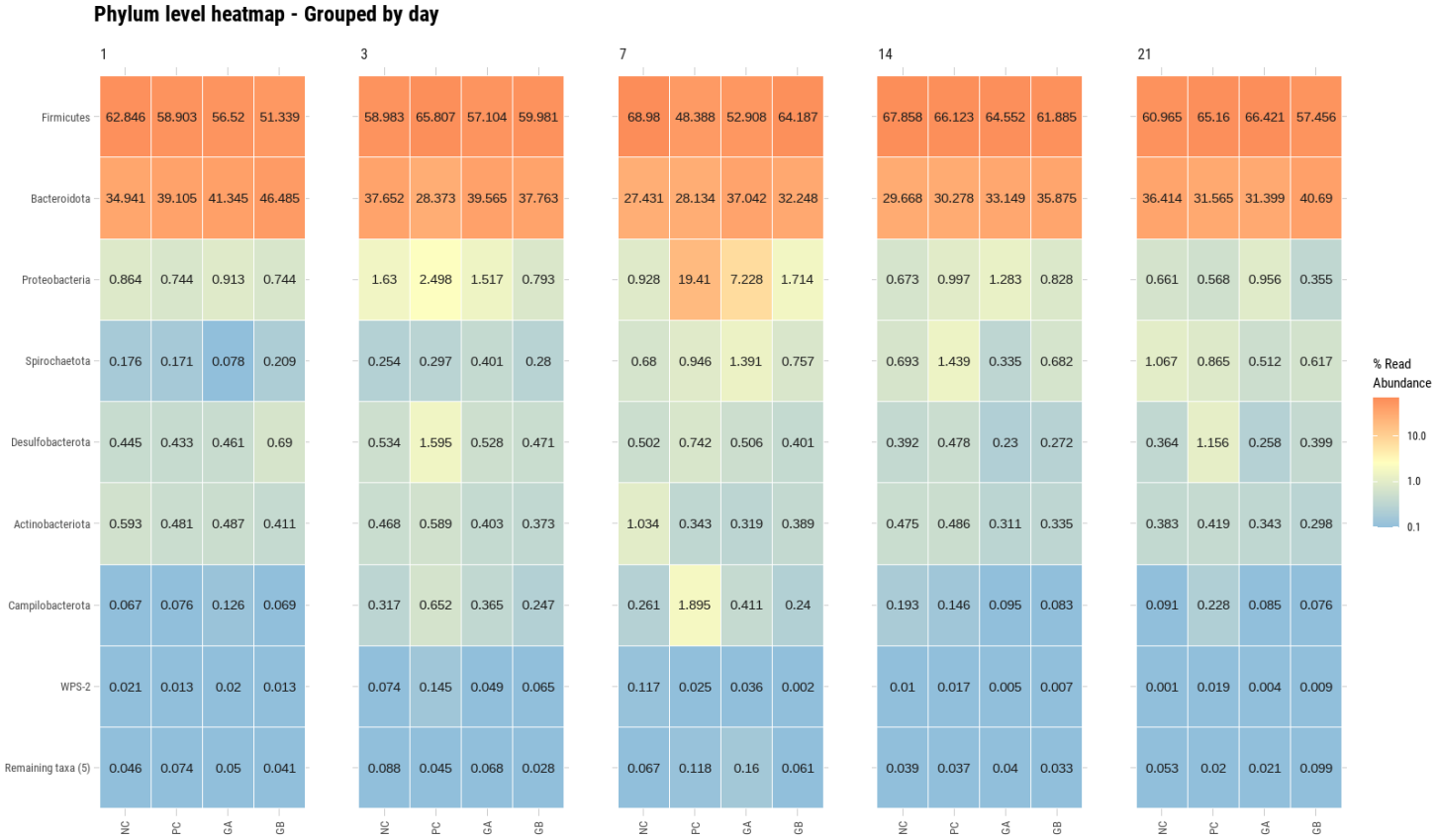

Fig S2 Relative abundance of bacterial genera within the feces of pigs challenged with enterotoxigenic *E. coli* (ETEC) F18 after weaning and supplemented with plant combinations. The ETEC F18 was administered orally on days 1 and 2. Values indicate mean relative abundance in percentage of the 20 dominant genera (Y-axis) across treatments (X-axis) and days postweaning (panel). NC: non-challenge, standard diet (n=8); PC: challenged, standard diet (0-7d, n=8; 7-21d, n=7); GA: challenged, Garlic + Apple pomace (3%+3%; 0-7d, n=8; 7-21d, n=6); GB: challenged, Garlic + Blackcurrant (3%+3%; n=8).

A

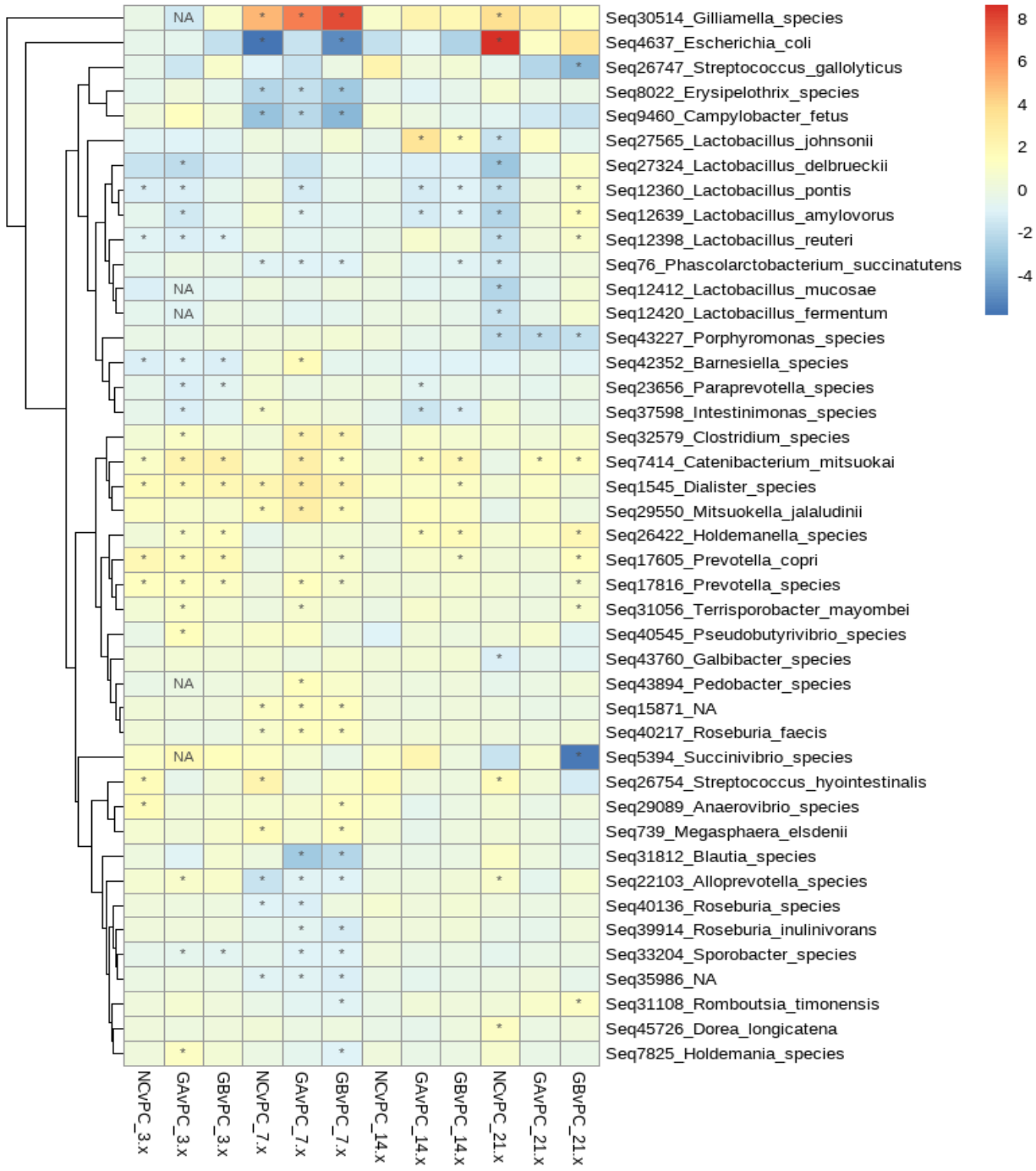

Fig S3 Differential abundance of bacterial genera within the feces of pigs challenged with enterotoxigenic E. coli (ETEC) F18 after weaning and supplemented with plant combinations. The ETEC F18 was administered orally on days 1 and 2. A: log2 fold-change difference from PC. (*): adj-P < 0.05 pairwise comparison against PC within day. NA: P value no estimable. B: DESeq2 normalized counts of differentially abundant genera. Only differentially abundant taxa (log2FC > 2; P < 0.05) are represented in the figures. NC: non-challenge, standard diet (n=8); PC: challenged, standard diet (0-7d, n=8; 7-21d, n=7); GA: challenged, Garlic + Apple pomace (3%+3%; 0-7d, n=8; 7-21d, n=6); GB: challenged, Garlic + Blackcurrant (3%+3%; n=8).

B

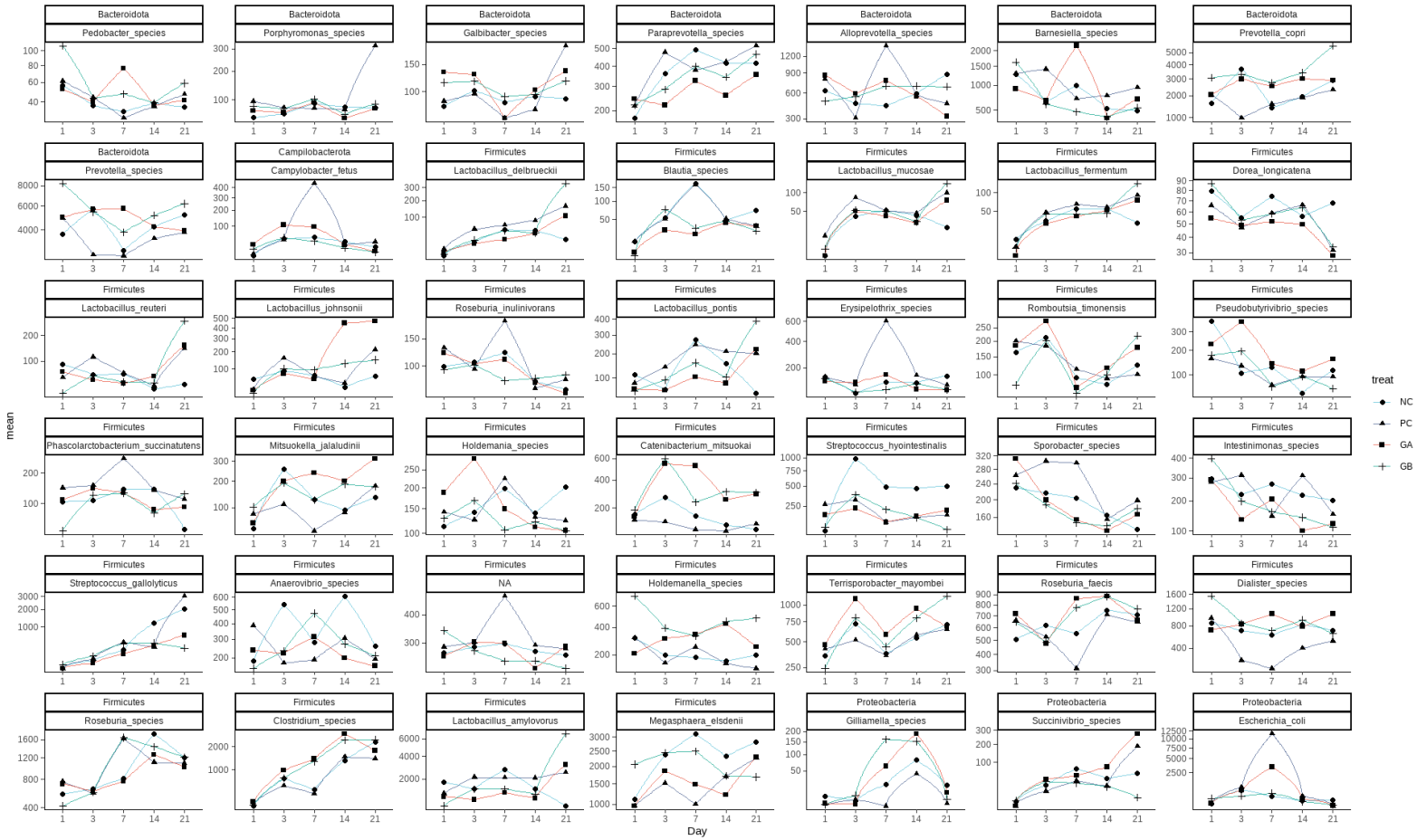

Fig S3 Differential abundance of bacterial genera within the feces of pigs challenged with enterotoxigenic E. coli (ETEC) F18 after weaning and supplemented with plant combinations. The ETEC F18 was administered orally on days 1 and 2. A: log2 fold-change difference from PC. (*): adj-P < 0.05 pairwise comparison against PC within day. NA: P value no estimable. B: DESeq2 normalized counts of differentially abundant genera. Only differentially abundant taxa (log2FC > 2; P < 0.05) are represented in the figures. NC: non-challenge, standard diet (n=8); PC: challenged, standard diet (0-7d, n=8; 7-21d, n=7); GA: challenged, Garlic + Apple pomace (3%+3%; 0-7d, n=8; 7-21d, n=6); GB: challenged, Garlic + Blackcurrant (3%+3%; n=8).

STUDY-II

Effects of Garlic with Apple Pomace or Blackcurrant Supplementation on the Gastrointestinal Microbial Ecosystem in Organic Pigs After Weaning

Kevin Jerez-Bogota, Martin Jensen, Ole Højberg and Nuria Canibe

Manuscript ready for submission to: Animal Microbiome.

Effects of Garlic with Apple Pomace or Blackcurrant Supplementation on the Gastrointestinal Microbial Ecosystem in Organic Pigs After Weaning

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6 **Keywords**: *apple pomace, blackcurrant, garlic, gastrointestinal, microbiota, microbial metabolites,*
7 *organic pigs, postweaning*

The printed version of this thesis includes the unpublished manuscript on pages 109 to 145. These pages have been omitted from the electronic version to prevent potential conflicts with future publication. For information regarding the manuscript's publication status, please contact the author.

STUDY-III

Efficacy of Medium Chain Fatty Acids and Essential Oils Against Necrotic Enteritis in Broilers

Kevin Jerez-Bogota, Martin Jensen, Ole Højberg, Nuria Canibe and Ricarda Engberg

Manuscript ready for submission to: Poultry Science.

Efficacy of Medium Chain Fatty Acids and Essential Oils Against Necrotic Enteritis in Broilers

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Scientific section: Health and Disease

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The printed version of this thesis includes the unpublished manuscript on pages 148 to 192. These pages have been omitted from the electronic version to prevent potential conflicts with future publication. For information regarding the manuscript's publication status, please contact the author.

BIBLIOGRAPHY

The specific reference list from each manuscript is listed within the manuscript document

Abd El-Hack, M. E., El-Saadony, M. T., Elbestawy, A. R., El-Shall, N. A., Saad, A. M., Salem, H. M., El-Tahan, A. M., Khafaga, A. F., Taha, A. E., AbuQamar, S. F. and El-Tarabily, K. A. (2022) 'Necrotic enteritis in broiler chickens: disease characteristics and prevention using organic antibiotic alternatives – a comprehensive review', *Poultry Science,* 101(2), pp. 101590. DOI: 10.1016/j.psj.2021.101590.

Abd El-Hack, M. E., El-Saadony, M. T., Shafi, M. E., Qattan, S. Y. A., Batiha, G. E., Khafaga, A. F., Abdel-Moneim, A.-M. E. and Alagawany, M. (2020) 'Probiotics in poultry feed: A comprehensive review', *Journal of Animal Physiology and Animal Nutrition,* 104(6), pp. 1835-1850. DOI: 10.1111/jpn.13454.

Abdelli, N., Pérez, J. F., Vilarrasa, E., Cabeza Luna, I., Melo-Duran, D., D'Angelo, M. and Solà-Oriol, D. (2020) 'Targeted-release organic acids and essential oils improve performance and digestive function in broilers under a necrotic enteritis challenge', *Animals,* 10(2), pp. 259.

Abdelli, N., Pérez, J.F., Vilarrasa, E., Luna, I.C., Melo-Duran, D., D'Angelo, M. and Solà-Oriol, D., 2020. Targeted-Release Organic Acids and Essential Oils Improve Performance and Digestive Function in Broilers under a Necrotic Enteritis Challenge. *Animals*, 10(2), p.259. DOI10.3390/ani10020259.

Abdelli, N., Solà-Oriol, D. and Pérez, J. F. (2021) 'Phytogenic Feed Additives in Poultry: Achievements, Prospective and Challenges', *Animals (Basel),* 11(12). DOI 10.3390/ani11123471.

Abildgaard, L., Hojberg, O., Schramm, A., Balle, K. M. and Engberg, R. M. (2010a) 'The effect of feeding a commercial essential oil product on Clostridium perfringens numbers in the intestine of broiler chickens measured by real-time PCR targeting the α-toxin-encoding gene (plc)', *Animal Feed Science and Technology,* 157(3), pp. 181-189. DOI: 10.1016/j.anifeedsci.2010.03.010.

Abildgaard, L., Sondergaard, T. E., Engberg, R. M., Schramm, A. and Højberg, O. (2010b) 'In vitro production of necrotic enteritis toxin B, NetB, by *netB*-positive and *netB*-negative Clostridium perfringens originating from healthy and diseased broiler chickens', *Veterinary microbiology,* 144(1-2), pp. 231-235.

Adewole, D., Kim, I. and Nyachoti, C. (2016) 'Gut health of pigs: challenge models and response criteria with a critical analysis of the effectiveness of selected feed additives—a review', *Asian-Australasian journal of animal sciences,* 29(7), pp. 909.

Ahmed Khan, R. and van Vuuren, S. F. (2021) 'Essential oil combinations against Clostridium perfringens and Clostridium septicum - the causative agents of gas gangrene', *Journal of Applied Microbiology,* 131(3), pp. 1177-1192. DOI: 10.1111/jam.15029.

Ahmed, S., Hwang, J., Hoon, J., Mun, H. and Yang, C. (2014) 'Comparison of single and blend acidifiers as alternative to antibiotics on growth performance, fecal microflora, and humoral immunity in weaned piglets', *Asian-Australasian journal of animal sciences,* 27(1), pp. 93.

Alibi, S., Crespo, D. and Navas, J. (2021) 'Plant-Derivatives Small Molecules with Antibacterial Activity', *Antibiotics,* 10(3), pp. 231.

Allwood, J. W., Woznicki, T. L., Xu, Y., Foito, A., Aaby, K., Sungurtas, J., Freitag, S., Goodacre, R., Stewart, D., Remberg, S. F., Heide, O. M. and Sønsteby, A. (2019) 'Application of HPLC–PDA–MS metabolite profiling to investigate the effect of growth temperature and day length on blackcurrant fruit', *Metabolomics,* 15(1), pp. 12. DOI: 10.1007/s11306-018-1462-5.

Alnassan, A. A., Kotsch, M., Shehata, A. A., Krüger, M., Daugschies, A. and Bangoura, B. (2014) 'Necrotic enteritis in chickens: development of a straightforward disease model system', *Veterinary Record,* 174(22), pp. 555-555. DOI: 10.1136/vr.102066.

Álvarez-Martínez, F. J., Barrajón-Catalán, E., Herranz-López, M. and Micol, V. (2021) 'Antibacterial plant compounds, extracts and essential oils: An updated review on their effects and putative mechanisms of action', *Phytomedicine,* 90, pp. 153626. DOI: 10.1016/j.phymed.2021.153626.

Amri, I. N. (2011) 'The Lauric (Coconut and Palm Kernel) Oils', *Vegetable Oils in Food Technology*, pp. 169-197.

Ankri, S. and Mirelman, D. (1999) 'Antimicrobial properties of allicin from garlic', *Microbes and Infection,* 1(2), pp. 125-129. DOI: 10.1016/S1286-4579(99)80003-3.

Annett, C., Viste, J., Chirino-Trejo, M., Classen, H., Middleton, D. and Simko, E. (2002) 'Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of Clostridium perfringens type A', *Avian Pathology,* 31(6), pp. 598-601.

Antonic, B., Jancikova, S., Dordevic, D. and Tremlova, B. (2020a) 'Apple pomace as food fortification ingredient: A systematic review and meta-analysis', *Journal of Food Science,* 85(10), pp. 2977-2985. DOI: 10.1111/1750-3841.15449.

Antonic, B., Jancikova, S., Dordevic, D. and Tremlova, B. (2020b) 'Apple pomace as food fortification ingredient: A systematic review and meta-analysis', *Journal of food science,* 85(10), pp. 2977-2985.

Antonissen, G., Croubels, S., Pasmans, F., Ducatelle, R., Eeckhaut, V., Devreese, M., Verlinden, M., Haesebrouck, F., Eeckhout, M., De Saeger, S., Antlinger, B., Novak, B., Martel, A. and Van Immerseel, F. (2015) 'Fumonisins affect the intestinal microbial homeostasis in broiler chickens, predisposing to necrotic enteritis', *Vet Res,* 46(1), pp. 98. DOI: 10.1186/s13567-015-0234-8.

Ao, W., Cheng, M., Chen, Y., Sun, J., Zhang, C., Zhao, X., Liu, M. and Zhou, B. (2022) 'Fermented Apple Pomace Improves Plasma Biochemical and Antioxidant Indicators and Fecal Microbiota of Weaned Pigs', *Agriculture,* 12(10), pp. 1603. Available at: https://www.mdpi.com/2077-0472/12/10/1603.

Arora, D. S. and Kaur, J. (1999) 'Antimicrobial activity of spices', *International Journal of Antimicrobial Agents,* 12(3), pp. 257-262. DOI: 10.1016/S0924-8579(99)00074-6.

Arraibi, A. A., Liberal, Â., Dias, M. I., Alves, M. J., Ferreira, I. C. F. R., Barros, L. and Barreira, J. C. M. (2021) 'Chemical and Bioactive Characterization of Spanish and Belgian Apple Pomace for Its Potential Use as a Novel Dermocosmetic Formulation', *Foods,* 10(8), pp. 1949. Available at: https://www.mdpi.com/2304-8158/10/8/1949.

Ayrle, H., Mevissen, M., Kaske, M., Nathues, H., Gruetzner, N., Melzig, M. and Walkenhorst, M. (2016) 'Medicinal plants – prophylactic and therapeutic options for gastrointestinal and respiratory diseases in calves and piglets? A systematic review', *BMC Veterinary Research,* 12(1), pp. 89. DOI: 10.1186/s12917-016-0714-8.

Ayrle, H., Nathues, H., Bieber, A., Durrer, M., Quander, N., Mevissen, M. and Walkenhorst, M. (2019) 'Placebo-controlled study on the effects of oral administration of Allium sativum L in postweaning piglets', *Veterinary Record,* 184(10), pp. 316-316. DOI: 10.1136/vr.105131.

Bailey, M. and Stokes, C. (2022) *Understanding gut microbiomes as targets for improving pig gut health.* Burleigh Dodds Science Publishing.

Bailey, M., Thomas, A., Francis, O., Stokes, C. and Smidt, H. (2019) 'The dark side of technological advances in analysis of microbial ecosystems', *Journal of Animal Science and Biotechnology,* 10(1), pp. 49. DOI: 10.1186/s40104-019-0357-2.

Baptista-Silva, S., Borges, S., Ramos, O. L., Pintado, M. and Sarmento, B. (2020) 'The progress of essential oils as potential therapeutic agents: a review', *Journal of Essential Oil Research,* 32(4), pp. 279-295. DOI: 10.1080/10412905.2020.1746698.

Bar, M., Binduga, U. E. and Szychowski, K. A. (2022) 'Methods of Isolation of Active Substances from Garlic (Allium sativum L.) and Its Impact on the Composition and Biological Properties of Garlic Extracts', *Antioxidants (Basel),* 11(7). DOI: 10.3390/antiox11071345.

Barbara, A. J., Trinh, H. T., Glock, R. D. and Glenn Songer, J. (2008) 'Necrotic enteritis-producing strains of Clostridium perfringens displace non-necrotic enteritis strains from the gut of chicks', *Veterinary Microbiology,* 126(4), pp. 377-382. DOI: 10.1016/j.vetmic.2007.07.019.

Barba-Vidal, E., Martín-Orúe, S. M. and Castillejos, L. (2018) 'Review: Are we using probiotics correctly in post-weaning piglets?', *animal,* 12(12), pp. 2489-2498. DOI: 10.1017/S1751731118000873.

Barros, M. M., Castro, J., Araújo, D., Campos, A. M., Oliveira, R., Silva, S., Outor-Monteiro, D. and Almeida, C. (2023) 'Swine Colibacillosis: Global Epidemiologic and Antimicrobial Scenario', *Antibiotics,* 12(4), pp. 682. Available at: https://www.mdpi.com/2079-6382/12/4/682.

Bars-Cortina, D., Moratalla-Navarro, F., García-Serrano, A., Mach, N., Riobó-Mayo, L., Vea-Barbany, J., Rius-Sansalvador, B., Murcia, S., Obón-Santacana, M. and Moreno, V. (2023) 'Improving Species Level-taxonomic Assignment from 16S rRNA Sequencing Technologies', *Current Protocols,* 3(11), pp. e930. DOI: 10.1002/cpz1.930.

Bartkiene, E., Bartkevics, V., Lele, V., Pugajeva, I., Zavistanaviciute, P., Zadeike, D. and Juodeikiene, G. (2019) 'Application of antifungal lactobacilli in combination with coatings based on apple processing by-products as a bio-preservative in wheat bread production', *Journal of food science and technology,* 56, pp. 2989-3000.

Bassolé, I. H. N. and Juliani, H. R. (2012) 'Essential Oils in Combination and Their Antimicrobial Properties', *Molecules,* 17(4), pp. 3989-4006. Available at: https://www.mdpi.com/1420-3049/17/4/3989.

Bastiaanssen, T. F. S., Gururajan, A., van de Wouw, M., Moloney, G. M., Ritz, N. L., Long-Smith, C. M., Wiley, N. C., Murphy, A. B., Lyte, J. M., Fouhy, F., Stanton, C., Claesson, M. J., Dinan, T. G. and Cryan, J. F. (2021) 'Volatility as a Concept to Understand the Impact of Stress on the Microbiome', *Psychoneuroendocrinology,* 124, pp. 105047. DOI: 10.1016/j.psyneuen.2020.105047.

Bellerose, M., Fravalo, P., Mainville, I., Arcand, Y. and Thibodeau, A. (2023) 'A short-term bioreactor assay to assess the effect of essential oils on a microbiota derived from piglet's intestinal content', *Acta Veterinaria Scandinavica,* 65(1), pp. 17. DOI: 10.1186/s13028-023-00679-w.

Bhatwalkar, S. B., Gound, S. S., Mondal, R., Srivastava, R. K. and Anupam, R. (2019) 'Anti-biofilm and Antibacterial Activity of Allium sativum Against Drug Resistant Shiga-Toxin Producing Escherichia coli (STEC) Isolates

from Patient Samples and Food Sources', *Indian Journal of Microbiology,* 59(2), pp. 171-179. DOI: 10.1007/s12088-019-00784-3.

Bhatwalkar, S. B., Mondal, R., Krishna, S. B. N., Adam, J. K., Govender, P. and Anupam, R. (2021) 'antibacterial properties of organosulfur compounds of garlic (Allium sativum)', *Frontiers in Microbiology,* 12.

Bhushan, S., Kalia, K., Sharma, M., Singh, B. and Ahuja, P. S. (2008) 'Processing of Apple Pomace for Bioactive Molecules', *Critical Reviews in Biotechnology,* 28(4), pp. 285-296. DOI: 10.1080/07388550802368895.

Blair, R. (2018) 'Nutrition and feeding of organic pigs', *Nutrition and feeding of organic pigs.,* (Ed. 2).

Blavi, L., Solà-Oriol, D., Llonch, P., López-Vergé, S., Martín-Orúe, S. M. and Pérez, J. F. (2021) 'Management and Feeding Strategies in Early Life to Increase Piglet Performance and Welfare around Weaning: A Review', *Animals,* 11(2), pp. 302. Available at: https://www.mdpi.com/2076-2615/11/2/302.

Bonetti, A., Tugnoli, B., Piva, A. and Grilli, E. (2021) 'Towards Zero Zinc Oxide: Feeding Strategies to Manage Post-Weaning Diarrhea in Piglets', *Animals,* 11(3), pp. 642. Available at: https://www.mdpi.com/2076-2615/11/3/642.

Bordonaba, J. G. and Terry, L. A. (2008) 'Biochemical Profiling and Chemometric Analysis of Seventeen UK-Grown Black Currant Cultivars', *Journal of Agricultural and Food Chemistry,* 56(16), pp. 7422-7430. DOI: 10.1021/jf8009377.

Bordonaba, J. G. and Terry, L. A. (2010) 'Manipulating the taste-related composition of strawberry fruits (Fragaria× ananassa) from different cultivars using deficit irrigation', *Food Chemistry,* 122(4), pp. 1020-1026.

Borlinghaus, J., Albrecht, F., Gruhlke, M. C. H., Nwachukwu, I. D. and Slusarenko, A. J. (2014) 'Allicin: Chemistry and Biological Properties', *Molecules,* 19(8), pp. 12591-12618. Available at: https://www.mdpi.com/1420-3049/19/8/12591.

Borlinghaus, J., Foerster , J., Kappler, U., Antelmann, H., Noll, U., Gruhlke, M. C. H. and Slusarenko, A. J. (2021) 'Allicin, the Odor of Freshly Crushed Garlic: A Review of Recent Progress in Understanding Allicin's Effects on Cells', *Molecules,* 26(6), pp. 1505. Available at: https://www.mdpi.com/1420-3049/26/6/1505.

Bruna, L., Draye, M., Cravotto, G. and Chatel, G. (2024) 'Waste Management of Apple Pomace: Extraction of Antimicrobial Molecules Using Green Technologies', *Waste and Biomass Valorization*. DOI: 10.1007/s12649-024-02432-4.

Brüssow, H. (2015) 'Growth promotion and gut microbiota: insights from antibiotic use', *Environmental Microbiology,* 17(7), pp. 2216-2227. DOI: 10.1111/1462-2920.12786.

Caesar, L. K. and Cech, N. B. (2019) 'Synergy and antagonism in natural product extracts: When 1+ 1 does not equal 2', *Natural product reports,* 36(6), pp. 869-888.

Cai, Y., Wang, R., Pei, F. and Liang, B.-B. (2007) 'Antibacterial Activity of Allicin Alone and in Combination with β-Lactams against Staphylococcus spp. and Pseudomonas aeruginosa', *The Journal of Antibiotics,* 60(5), pp. 335-338. DOI: 10.1038/ja.2007.45.

Canibe, N., Højberg, O., Grevsen, K. and Jensen, M. 'Plants with anitimicrobial effects to combat post-weaning diarrhea in piglets - Allicin and acid-containing species'. *14th International Symposium on Digestive Physiology of Pigs*, Australia, 8.

Canibe, N., Højberg, O., Kongsted, H., Vodolazska, D., Lauridsen, C., Nielsen, T. S. and Schönherz, A. A. (2022) 'Review on Preventive Measures to Reduce Post-Weaning Diarrhoea in Piglets', *Animals,* 12(19), pp. 2585. Available at: https://www.mdpi.com/2076-2615/12/19/2585.

Cañizares, P., Gracia, I., Gómez, L. A., de Argila, C. M., Boixeda, D., García, A. and de Rafael, L. (2004a) 'Allyl-thiosulfinates, the Bacteriostatic Compounds of Garlic against Helicobacter pylori', *Biotechnology Progress,* 20(1), pp. 397-401. DOI: 10.1021/bp034143b.

Cañizares, P., Gracia, I., Gómez, L. A., García, A., De Argila, C. M., Boixeda, D. and de Rafael, L. (2004b) 'Thermal degradation of allicin in garlic extracts and its implication on the inhibition of the in-vitro growth of Helicobacter pylori', *Biotechnology progress,* 20(1), pp. 32-37.

Cargnin, S. T. and Gnoatto, S. B. (2017) 'Ursolic acid from apple pomace and traditional plants: A valuable triterpenoid with functional properties', *Food Chemistry,* 220, pp. 477-489. DOI: 10.1016/j.foodchem.2016.10.029.

Carlsson, F. and Råberg, L. (2024) 'The germ theory revisited: A noncentric view on infection outcome', *Proceedings of the National Academy of Sciences,* 121(17), pp. e2319605121. DOI: 10.1073/pnas.2319605121.

Casella, S., Leonardi, M., Melai, B., Fratini, F. and Pistelli, L. (2013) 'The Role of Diallyl Sulfides and Dipropyl Sulfides in the In Vitro Antimicrobial Activity of the Essential Oil of Garlic, Allium sativum L., and Leek, Allium porrum L', *Phytotherapy Research,* 27(3), pp. 380-383. DOI: 10.1002/ptr.4725.

Cavallito, C. J. and Bailey, J. H. (1944a) 'Allicin, the antibacterial principle of Allium sativum. I. Isolation, physical properties and antibacterial action', *Journal of the American Chemical Society,* 66(11), pp. 1950-1951.

Cavallito, C. J. and Bailey, J. H. (1944b) 'Preliminary Note on the Inactivation of Antibiotics', *Science,* 100(2600), pp. 390-390. DOI: 10.1126/science.100.2600.390.a.

Chapman, H. D., Jeffers, T. K. and Williams, R. B. (2010) 'Forty years of monensin for the control of coccidiosis in poultry', *Poultry Science,* 89(9), pp. 1788-1801. DOI: 10.3382/ps.2010-00931.

Chatterjee, S., Leach, S. T., Lui, K. and Mishra, A. (2024) 'Symbiotic symphony: Understanding host-microbiota dialogues in a spatial context', *Seminars in Cell & Developmental Biology,* 161-162, pp. 22-30. DOI: 10.1016/j.semcdb.2024.03.001.

Cheesman, M. J., Ilanko, A., Blonk, B. and Cock, I. E. (2017) 'Developing New Antimicrobial Therapies: Are Synergistic Combinations of Plant Extracts/Compounds with Conventional Antibiotics the Solution?', *Pharmacogn Rev,* 11(22), pp. 57-72. DOI: 10.4103/phrev.phrev_21_17.

Chen, J., Wang, F., Yin, Y. and Ma, X. (2021) 'The nutritional applications of garlic (Allium sativum) as natural feed additives in animals', *PeerJ,* 9, pp. e11934.

Choi, J., Wang, L., Liu, S., Lu, P., Zhao, X., Liu, H., Lahaye, L., Santin, E., Liu, S., Nyachoti, M. and Yang, C. (2020a) 'Effects of a microencapsulated formula of organic acids and essential oils on nutrient absorption, immunity, gut barrier function, and abundance of enterotoxigenic Escherichia coli F4 in weaned piglets challenged with E. coli F4', *J Anim Sci,* 98(9). DOI: 10.1093/jas/skaa259.

Choi, S.-H., Bong, Y.-S., Park, J. H. and Lee, K.-S. (2020b) 'Geographical origin identification of garlic cultivated in Korea using isotopic and multi-elemental analyses', *Food Control,* 111, pp. 107064. DOI: 10.1016/j.foodcont.2019.107064.

Choo, S., Chin, V. K., Wong, E. H., Madhavan, P., Tay, S. T., Yong, P. V. C. and Chong, P. P. (2020) 'Review: antimicrobial properties of allicin used alone or in combination with other medications', *Folia Microbiologica,* 65(3), pp. 451-465. DOI: 10.1007/s12223-020-00786-5.

Chouhan, S., Sharma, K. and Guleria, S. (2017) 'Antimicrobial Activity of Some Essential Oils—Present Status and Future Perspectives', *Medicines,* 4(3), pp. 58. Available at: https://www.mdpi.com/2305-6320/4/3/58.

Coles, M. E., Forga, A. J., Señas-Cuesta, R., Graham, B. D., Selby, C. M., Uribe Á, J., Martínez, B. C., Angel-Isaza, J. A., Vuong, C. N., Hernandez-Velasco, X., Hargis, B. M. and Tellez-Isaias, G. (2021) 'Assessment of Lippia origanoides Essential Oils in a Salmonella typhimurium, Eimeria maxima, and Clostridium perfringens Challenge Model to Induce Necrotic Enteritis in Broiler Chickens', *Animals (Basel),* 11(4). DOI: 10.3390/ani11041111.

Combrink, L., Humphreys, I. R., Washburn, Q., Arnold, H. K., Stagaman, K., Kasschau, K. D., Jolles, A. E., Beechler, B. R. and Sharpton, T. J. (2023) 'Best practice for wildlife gut microbiome research: A comprehensive review of methodology for 16S rRNA gene investigations', *Frontiers in Microbiology,* 14. DOI: 10.3389/fmicb.2023.1092216.

Cortez, R. E. and Gonzalez de Mejia, E. (2019) 'Blackcurrants (Ribes nigrum): A Review on Chemistry, Processing, and Health Benefits', *Journal of Food Science,* 84(9), pp. 2387-2401. DOI: 10.1111/1750-3841.14781.

Costa, C. M., de Carvalho, N. M., de Oliveira, D. L. and Madureira, A. R. (2024) 'A Critical Review on In Vitro and Ex Vivo Models of the Intestinal Epithelium of Humans and Monogastric Animals', *Gastrointestinal Disorders,* 6(1), pp. 337-358. Available at: https://www.mdpi.com/2624-5647/6/1/23.

Cromwell, G. L. (2013) 'Feed additives in swine diets', *Sustainable swine nutrition*, pp. 295-315.

Curtasu, M. V. and Nørskov, N. P. (2023) 'Comprehensive quantification of flavonoids and salicylic acid representative of Salix spp. using microLiquid Chromatography-Triple Quadrupole Mass Spectrometry: the importance of drying procedures and extraction solvent when performing classical solid-liquid extraction', *Journal of Chromatography A,* 1705, pp. 464139. DOI: 10.1016/j.chroma.2023.464139.

Dahiya, J. P., Hoehler, D., Wilkie, D. C., Van Kessel, A. G. and Drew, M. D. (2005) 'Dietary glycine concentration affects intestinal Clostridium perfringens and lactobacilli populations in broiler chickens', *Poult Sci,* 84(12), pp. 1875-85. DOI: 10.1093/ps/84.12.1875.

Dahiya, J. P., Wilkie, D. C., Van Kessel, A. G. and Drew, M. D. (2006) 'Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era', *Animal Feed Science and Technology,* 129(1), pp. 60-88. DOI: 10.1016/j.anifeedsci.2005.12.003.

Dahmer, P. L., DeRouchey, J. M., Gebhardt, J. T., Paulk, C. B. and Jones, C. K. (2023) 'Summary of methodology used in enterotoxigenic Escherichia coli (ETEC) challenge experiments in weanling pigs and quantitative assessment of observed variability', *Translational Animal Science,* 7(1). DOI: 10.1093/tas/txad083.

Dalgaard, T. S., Rebel, J. M. J., Bortoluzzi, C. and Kogut, M. H. (2022) 'Chapter 15 - Factors modulating the avian immune system', in Kaspers, B., Schat, K.A., Göbel, T.W. & Vervelde, L. (eds.) *Avian Immunology (Third Edition)*. Boston: Academic Press, pp. 419-435.

Daly, A. J., Baetens, J. M. and De Baets, B. (2018) 'Ecological diversity: measuring the unmeasurable', *Mathematics,* 6(7), pp. 119.

Danneskiold-Samsøe, N. B., Dias de Freitas Queiroz Barros, H., Santos, R., Bicas, J. L., Cazarin, C. B. B., Madsen, L., Kristiansen, K., Pastore, G. M., Brix, S. and Maróstica Júnior, M. R. (2019) 'Interplay between food and gut microbiota in health and disease', *Food Research International,* 115, pp. 23-31. DOI: 10.1016/j.foodres.2018.07.043.

Das, Q., Islam, M. R., Marcone, M. F., Warriner, K. and Diarra, M. S. (2017) 'Potential of berry extracts to control foodborne pathogens', *Food Control,* 73, pp. 650-662. DOI: 10.1016/j.foodcont.2016.09.019.

Das, T., Chatterjee, N., Capanoglu, E., Lorenzo, J. M., Das, A. K. and Dhar, P. (2023) 'The synergistic ramification of insoluble dietary fiber and associated non-extractable polyphenols on gut microbial population escorting alleviation of lifestyle diseases', *Food Chemistry: X*, pp. 100697.

Dayrit, F. M. (2014) 'Lauric acid is a medium-chain fatty acid, coconut oil is a medium-chain triglyceride', *Philippine Journal of Science,* 143(2), pp. 157-166.

de Carvalho, N. M., Oliveira, D. L., Saleh, M. A. D., Pintado, M. E. and Madureira, A. R. (2021) 'Importance of gastrointestinal in vitro models for the poultry industry and feed formulations', *Animal Feed Science and Technology,* 271, pp. 114730.

de Lange, C. F. M., Pluske, J., Gong, J. and Nyachoti, C. M. (2010) 'Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs', *Livestock Science,* 134(1), pp. 124-134. DOI: 10.1016/j.livsci.2010.06.117.

der Poel, A. F. B. v., Abdollahi, M. R., Cheng, H., Colovic, R., den Hartog, L. A., Miladinovic, D., Page, G., Sijssens, K., Smillie, J. F., Thomas, M., Wang, W., Yu, P. and Hendriks, W. H. (2020) 'Future directions of animal feed technology research to meet the challenges of a changing world', *Animal Feed Science and Technology,* 270, pp. 114692. DOI: 10.1016/j.anifeedsci.2020.114692.

Desbois, A. P. and Smith, V. J. (2010) 'Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential', *Applied microbiology and biotechnology,* 85, pp. 1629-1642.

Diaz Carrasco, J. M., Redondo, L. M., Redondo, E. A., Dominguez, J. E., Chacana, A. P. and Fernandez Miyakawa, M. E. (2016) 'Use of Plant Extracts as an Effective Manner to Control Clostridium perfringens</i> Induced Necrotic Enteritis in Poultry', *BioMed Research International,* 2016, pp. 3278359. DOI: 10.1155/2016/3278359.

Doern, C. D. (2014) 'When Does 2 Plus 2 Equal 5? A Review of Antimicrobial Synergy Testing', *Journal of Clinical Microbiology,* 52(12), pp. 4124-4128. DOI: 10.1128/jcm.01121-14.

Du, E. and Guo, Y. (2021) 'Dietary supplementation of essential oils and lysozyme reduces mortality and improves intestinal integrity of broiler chickens with necrotic enteritis', *Anim Sci J,* 92(1), pp. e13499. DOI: 10.1111/asj.13499.

Du, E., Gan, L., Li, Z., Wang, W., Liu, D. and Guo, Y. (2015) 'In vitro antibacterial activity of thymol and carvacrol and their effects on broiler chickens challenged with Clostridium perfringens', *Journal of animal science and biotechnology,* 6, pp. 1-12.

Du, E., Wang, W., Gan, L., Li, Z., Guo, S. and Guo, Y. (2016) 'Effects of thymol and carvacrol supplementation on intestinal integrity and immune responses of broiler chickens challenged with *Clostridium perfringens*', *Journal of Animal Science and Biotechnology,* 7(1), pp. 19. DOI: 10.1186/s40104-016-0079-7.

Duarte, A. S. R., Attauabi, M., Sandberg, M., Lindegaard, M., Sönksen, U. W. and Andersen, V. D. (2023a) 'DANMAP 2022: Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark'.

Duarte, M. E. and Kim, S. W. (2022) 'Significance of Mucosa-Associated Microbiota and Its Impacts on Intestinal Health of Pigs Challenged with F18+ E. coli', *Pathogens,* 11(5), pp. 589. Available at: https://www.mdpi.com/2076-0817/11/5/589.

Duarte, M. E., Garavito-Duarte, Y. and Kim, S. W. (2023b) 'Impacts of F18+ *Escherichia coli* on Intestinal Health of Nursery Pigs and Dietary Interventions', *Animals,* 13(17), pp. 2791. Available at: https://www.mdpi.com/2076-2615/13/17/2791.

Dufourny, S., Antoine, N., Pitchugina, E., Delcenserie, V., Godbout, S., Douny, C., Scippo, M.-L., Froidmont, E., Rondia, P., Wavreille, J. and Everaert, N. (2021) 'Apple Pomace and Performance, Intestinal Morphology and Microbiota of Weaned Piglets—A Weaning Strategy for Gut Health?', *Microorganisms,* 9(3), pp. 572..

Dufourny, S., Everaert, N., Lebrun, S., Douny, C., Scippo, M.-L., Li, B., Taminiau, B., Marzorati, M., Wavreille, J., Froidmont, E., Rondia, P. and Delcenserie, V. (2019) 'Baby-SPIME: A dynamic in vitro piglet model mimicking gut microbiota during the weaning process', *Journal of Microbiological Methods,* 167, pp. 105735. DOI: 10.1016/j.mimet.2019.105735.

Dufourny, S., Lebrun, S., Douny, C., Dubois, B., Scippo, M.-L., Wavreille, J., Rondia, P., Everaert, N. and Delcenserie, V. (2022) 'Apple Pomace Modulates the Microbiota and Increases the Propionate Ratio in an In Vitro Piglet Gastrointestinal Model', *Fermentation,* 8(8), pp. 408. Available at: https://www.mdpi.com/2311-5637/8/8/408.

Duncan, S. H., Louis, P., Thomson, J. M. and Flint, H. J. (2009) 'The role of pH in determining the species composition of the human colonic microbiota', *Environmental microbiology,* 11(8), pp. 2112-2122.

Edwards, S., Turpin, D. and Pluske, J. (2020) 'Weaning age and its long-term influence on health and performance', *The suckling and weaned piglet*: Wageningen Academic Publishers, pp. 1800-1806.

El Astal, Z. (2004) 'The inhibitory action of aqueous garlic extract on the growth of certain pathogenic bacteria', *European Food Research and Technology,* 218(5), pp. 460-464. DOI: 10.1007/s00217-003-0864-3.

EMA, C. f. M. P. f. V. U. and EFSA, P. o. B. H. (2017) 'EMA and EFSA Joint Scientific Opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety (RONAFA)', *EFSA Journal,* 15(1), pp. e04666.

Emami, N. K. and Dalloul, R. A. (2021) 'Centennial Review: Recent developments in host-pathogen interactions during necrotic enteritis in poultry', *Poultry Science,* 100(9), pp. 101330. DOI: 10.1016/j.psj.2021.101330.

Eriksen, E. Ø., Kudirkiene, E., Barington, K., Goecke, N. B., Blirup-Plum, S. A., Nielsen, J. P., Olsen, J. E., Jensen, H. E., Pankoke, K., Larsen, L. E., Liu, G. and Pedersen, K. S. (2023) 'An observational field study of porcine post-weaning diarrhea: clinical and microbiological findings, and fecal pH-measurements as a potential diagnostic tool', *Porcine Health Management,* 9(1), pp. 33. DOI: 10.1186/s40813-023-00325-x.

Escobar-Páramo, P., Clermont, O., Blanc-Potard, A.-B. a., Bui, H., Le Bouguénec, C. and Denamur, E. (2004) 'A Specific Genetic Background Is Required for Acquisition and Expression of Virulence Factors in Escherichia coli', *Molecular Biology and Evolution,* 21(6), pp. 1085-1094. DOI: 10.1093/molbev/msh118.

Eurostat (2024) *The fruit and vegetable sector in the EU: a statistical overview*. Available at: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=The_fruit_and_vegetable_sector_in_the_EU_-_a_statistical_overview&stable=0&redirect=no#Where_are_fruits_and_vegetables_grown_in_the_EU.3F_By_how_many_farms.3F_Over_what_area.3F (Accessed: April).

Ezzat, S. M., Salama, M., El Kersh, D. and Salem, M. (2022) 'Apple Pomace as a Source of Nutraceuticals', in Egbuna, C., Sawicka, B. & Khan, J. (eds.) *Food and Agricultural Byproducts as Important Source of Valuable Nutraceuticals*. Cham: Springer International Publishing, pp. 75-86.

Fairbrother, J. M., Nadeau, É. and Gyles, C. L. (2005) 'Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies', *Animal Health Research Reviews,* 6(1), pp. 17-39. DOI: 10.1079/AHR2005105.

Fan, M. Z. and Archbold, T. (2015) 'Novel and disruptive biological strategies for resolving gut health challenges in monogastric food animal production', *Animal Nutrition,* 1(3), pp. 138-143. DOI: 10.1016/j.aninu.2015.10.002.

Fankam, A. G. and Kuete, V. (2023) 'Chapter Four - Screening methods for antibacterial agents from plant source', in Kuete, V. (ed.) *Advances in Botanical Research*: Academic Press, pp. 61-79.

Farrag, H. A., Hosny, A. E.-D. M. S., Hawas, A. M., Hagras, S. A. A. and Helmy, O. M. (2019) 'Potential efficacy of garlic lock therapy in combating biofilm and catheter-associated infections; experimental studies on an animal model with focus on toxicological aspects', *Saudi Pharmaceutical Journal,* 27(6), pp. 830-840. DOI: 10.1016/j.jsps.2019.05.004.

Feldberg, R. S., Chang, S., Kotik, A., Nadler, M., Neuwirth, Z., Sundstrom, D. and Thompson, N. (1988) 'In vitro mechanism of inhibition of bacterial cell growth by allicin', *Antimicrobial agents and chemotherapy,* 32(12), pp. 1763-1768.

Feng, S., Eucker, T. P., Holly, M. K., Konkel, M. E., Lu, X. and Wang, S. (2014) 'Investigating the Responses of Cronobacter sakazakii to Garlic-Drived Organosulfur Compounds: a Systematic Study of Pathogenic-Bacterium Injury by Use of High-Throughput Whole-Transcriptome Sequencing and Confocal Micro-Raman Spectroscopy', *Applied and Environmental Microbiology,* 80(3), pp. 959-971. DOI: 10.1128/AEM.03460-13.

Figueiredo, A. C., Barroso, J. G., Pedro, L. G. and Scheffer, J. J. C. (2008) 'Factors affecting secondary metabolite production in plants: volatile components and essential oils', *Flavour and Fragrance Journal,* 23(4), pp. 213-226. DOI: 10.1002/ffj.1875.

Food and Agriculture Organization of the United Nations (FAO), 2023. Global Meat Production Statistics. Available at: https://www.fao.org/faostat/en/ [Accessed April 2024].

Forgie, A. J., Fouhse, J. M. and Willing, B. P. (2019) 'Diet-Microbe-Host Interactions That Affect Gut Mucosal Integrity and Infection Resistance', *Front Immunol,* 10, pp. 1802. DOI: 10.3389/fimmu.2019.01802.

Francis, D. H. (2002) 'Enterotoxigenic Escherichia coli infection in pigs and its diagnosis', *Journal of Swine Health and Production,* 10(4), pp. 171-175.

Franz, C. M., Baser, K. H. C. and Hahn-Ramssl, I. (2020) 'Chapter 3 - Herbs and aromatic plants as feed additives: Aspects of composition, safety, and registration rules', in Florou-Paneri, P., Christaki, E. & Giannenas, I. (eds.) *Feed Additives*: Academic Press, pp. 35-56.

Franz, C., Baser, K. and Windisch, W. (2010) 'Essential oils and aromatic plants in animal feeding–a European perspective. A review', *Flavour and Fragrance Journal,* 25(5), pp. 327-340.

Frederiksen, R. F., Slettemeås, J. S., Granstad, S., Lagesen, K., Pikkemaat, M. G., Urdahl, A. M. and Simm, R. (2024) 'Polyether ionophore resistance in a one health perspective', *Frontiers in Microbiology,* 15. Available at: https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2024.1347490.

Freese, E., Sheu, C. W. and Galliers, E. (1973) 'Function of Lipophilic Acids as Antimicrobial Food Additives', *Nature,* 241(5388), pp. 321-325. DOI: 10.1038/241321a0.

Fujisawa, H., Watanabe, K., Suma, K., Origuchi, K., Matsufuji, H., Seki, T. and Ariga, T. (2009) 'Antibacterial potential of garlic-derived allicin and its cancellation by sulfhydryl compounds', *Bioscience, biotechnology, and biochemistry,* 73(9), pp. 1948-1955.

Galbraith, H., Miller, T., Paton, A. and Thompson, J. (1971) 'Antibacterial activity of long chain fatty acids and the reversal with calcium, magnesium, ergocalciferol and cholesterol', *Journal of applied Bacteriology,* 34(4), pp. 803-813.

Gao, J., Yin, J., Xu, K., Li, T. and Yin, Y. (2019a) 'What Is the Impact of Diet on Nutritional Diarrhea Associated with Gut Microbiota in Weaning Piglets: A System Review', *BioMed Research International,* 2019, pp. 6916189. DOI: 10.1155/2019/6916189.

Gao, J., Zhang, H.J., Wu, S.G., Yu, S.H., Yoon, I., Moore, D. and Gao, Y.P., 2019. Encapsulated blends of essential oils and organic acids in broilers challenged with necrotic enteritis. *Czech Journal of Animal Science*, 64(3), pp.79-86. DOI:10.17221/172/2018-CJAS.

Gao, Y.-Y., Zhang, X.-L., Xu, L.-H., Peng, H., Wang, C.-K. and Bi, Y.-Z. (2019b) 'Encapsulated blends of essential oils and organic acids improved performance, intestinal morphology, cecal microflora, and jejunal enzyme activity of broilers', *Czech Journal of Animal Science,* 64(5).

Gaskins, H. R., Collier, C. T. and Anderson, D. B. (2002) 'ANTIBIOTICS AS GROWTH PROMOTANTS:MODE OF ACTION', *Animal Biotechnology,* 13(1), pp. 29-42. DOI: 10.1081/ABIO-120005768.

Geary, N. (2013) 'Understanding synergy', *American Journal of Physiology-Endocrinology and Metabolism,* 304(3), pp. E237-E253. DOI: 10.1152/ajpendo.00308.2012.

Gebhardt, J., Tokach, M., Dritz, S., Cernicchiaro, N., Dixon, A., Renter, D., Woodworth, J., DeRouchey, J. and Goodband, R. (2020) 'Practical Application of Sample Size Determination Models for Assessment of Mortality Outcomes in Swine Field Trials', *Kansas Agricultural Experiment Station Research Reports,* 6(10), pp. 23.

Ghany, S. S. H. A. E., Ibrahem, R. A., El-Gendy, A. O., El-Baky, R. M. A., Mustafa, A. and Azmy, A. F. (2024) 'Novel synergistic interactions between monolaurin, a mono-acyl glycerol and β lactam antibiotics against Staphylococcus aureus: an in vitro study', *BMC Infectious Diseases,* 24(1), pp. 379. DOI: 10.1186/s12879-024-09261-9.

Gharib-Naseri, K., Kheravii, S. K., Keerqin, C., Morgan, N., Swick, R. A., Choct, M. and Wu, S. B. (2019) 'Two different Clostridium perfringens strains produce different levels of necrotic enteritis in broiler chickens', *Poultry Science,* 98(12), pp. 6422-6432. DOI: 10.3382/ps/pez480.

Gilbert, M. S., Ijssennagger, N., Kies, A. K. and van Mil, S. W. (2018) 'Protein fermentation in the gut; implications for intestinal dysfunction in humans, pigs, and poultry', *American Journal of Physiology-Gastrointestinal and Liver Physiology*.

Gill, A. O. and Holley, R. A. (2006) 'Inhibition of membrane bound ATPases of Escherichia coli and Listeria monocytogenes by plant oil aromatics', *International Journal of Food Microbiology,* 111(2), pp. 170-174. DOI: 10.1016/j.ijfoodmicro.2006.04.046.

Girard, M. and Bee, G. (2020) 'Invited review: Tannins as a potential alternative to antibiotics to prevent coliform diarrhea in weaned pigs', *animal,* 14(1), pp. 95-107. DOI: 10.1017/S1751731119002143.

Golubkina, N., Kharchenko, V., Bogachuk, M., Koshevarov, A., Sheshnitsan, S., Kosheleva, O., Pirogov, N. and Caruso, G. (2022) 'Biochemical Characteristics and Elemental Composition Peculiarities of Rheum tataricum L. in Semi-Desert Conditions and of European Garden Rhubarb', *International Journal of Plant Biology,* 13(3), pp. 368-380. Available at: https://www.mdpi.com/2037-0164/13/3/31.

Gomez-Osorio, L.-M., Yepes-Medina, V., Ballou, A., Parini, M. and Angel, R. (2021) 'Short and Medium Chain Fatty Acids and Their Derivatives as a Natural Strategy in the Control of Necrotic Enteritis and Microbial Homeostasis in Broiler Chickens', *Frontiers in Veterinary Science,* 8. DOI: 10.3389/fvets.2021.773372.

Gopalan, A., Reuben, S. C., Ahmed, S., Darvesh, A. S., Hohmann, J. and Bishayee, A. (2012) 'The health benefits of blackcurrants', *Food Funct,* 3(8), pp. 795-809. DOI: 10.1039/c2fo30058c.

Graham, M. L. and Prescott, M. J. (2015) 'The multifactorial role of the 3Rs in shifting the harm-benefit analysis in animal models of disease', *European Journal of Pharmacology,* 759, pp. 19-29. DOI: 10.1016/j.ejphar.2015.03.040.

Gresse, R., Chaucheyras-Durand, F., Denis, S., Beaumont, M., Van de Wiele, T., Forano, E. and Blanquet-Diot, S. (2021a) 'Weaning-associated feed deprivation stress causes microbiota disruptions in a novel mucin-containing in vitro model of the piglet colon (MPigut-IVM)', *J Anim Sci Biotechnol,* 12(1), pp. 75. DOI: 10.1186/s40104-021-00584-0.

Gresse, R., Chaucheyras-Durand, F., Fleury, M. A., Van de Wiele, T., Forano, E. and Blanquet-Diot, S. (2017) 'Gut Microbiota Dysbiosis in Postweaning Piglets: Understanding the Keys to Health', *Trends in Microbiology,* 25(10), pp. 851-873. DOI: 10.1016/j.tim.2017.05.004.

Gresse, R., Chaucheyras-Durand, F., Garrido, J. J., Denis, S., Jiménez-Marín, A., Beaumont, M., Van de Wiele, T., Forano, E. and Blanquet-Diot, S. (2021b) 'Pathogen Challenge and Dietary Shift Alter Microbiota Composition and Activity in a Mucin-Associated in vitro Model of the Piglet Colon (MPigut-IVM) Simulating Weaning Transition', *Frontiers in Microbiology,* 12. DOI: 10.3389/fmicb.2021.703421.

Gruhlke, M. C. H., Hemmis, B., Noll, U., Wagner, R., Lühring, H. and Slusarenko, A. J. (2015) 'The defense substance allicin from garlic permeabilizes membranes of Beta vulgaris, Rhoeo discolor, Chara corallina and artificial lipid bilayers', *Biochimica et Biophysica Acta (BBA) - General Subjects,* 1850(4), pp. 602-611. DOI: 10.1016/j.bbagen.2014.11.020.

Guil-Guerrero, J. L., Ramos, L., Moreno, C., Zuniga-Paredes, J. C., Carlosama-Yepez, M. and Ruales, P. (2016) 'Plant-food by-products to improve farm-animal health', *Animal Feed Science and Technology,* 220, pp. 121-135. DOI: 10.1016/j.anifeedsci.2016.07.016.

Hanczakowska, E. (2017) 'The use of medium-chain fatty acids in piglet feeding–a review', *Annals of animal science,* 17(4), pp. 967-977.

Hansen, L., Lauridsen, C., Nielsen, B., Jørgensen, L. and Canibe, N. (2022) 'Impact of early inoculation of probiotics to suckling piglets on postweaning diarrhoea–a challenge study with Enterotoxigenic E. Coli F18', *animal,* 16(11), pp. 100667.

He, L., Zhao, X., Li, J. and Yang, C. (2022) 'Post-weaning diarrhea and use of feedstuffs in pigs', *Animal Frontiers,* 12(6), pp. 41-52. DOI: 10.1093/af/vfac079.

Hedemann, M. S. and Bach Knudsen, K. E. (2010) 'A diet containing dried chicory root does not protect against post-weaning diarrhoea in an E. coli challenge model using piglets weaned at 7weeks of age', *Livestock Science,* 133(1), pp. 232-235. DOI: 10.1016/j.livsci.2010.06.073.

Heegaard, P. M. H., Stockmarr, A., Piñeiro, M., Carpintero, R., Lampreave, F., Campbell, F. M., Eckersall, P. D., Toussaint, M. J. M., Gruys, E. and Sorensen, N. S. (2011) 'Optimal combinations of acute phase proteins for detecting infectious disease in pigs', *Veterinary Research,* 42(1), pp. 50. DOI: 10.1186/1297-9716-42-50.

Hein, M. Y., Sharma, K., Cox, J. and Mann, M. (2013) 'Chapter 1 - Proteomic Analysis of Cellular Systems', in Walhout, A.J.M., Vidal, M. & Dekker, J. (eds.) *Handbook of Systems Biology*. San Diego: Academic Press, pp. 3-25.

Heo, J. M., Opapeju, F. O., Pluske, J. R., Kim, J. C., Hampson, D. J. and Nyachoti, C. M. (2013) 'Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds', *Journal of Animal Physiology and Animal Nutrition,* 97(2), pp. 207-237. DOI: 10.1111/j.1439-0396.2012.01284.x.

Herman, A. and Herman, A. P. (2023) 'Herbal products and their active constituents used alone and in combination with antibiotics against multidrug-resistant bacteria', *Planta Medica,* 89(02), pp. 168-182.

Hirata, S., Abdelrahman, M., Yamauchi, N. and Shigyo, M. (2016) 'Characteristics of chemical components in genetic resources of garlic Allium sativum collected from all over the world', *Genetic Resources and Crop Evolution,* 63(1), pp. 35-45. DOI: 10.1007/s10722-015-0233-7.

Hirshfield, I. N., Terzulli, S. and O'Byrne, C. (2003) 'Weak organic acids: a panoply of effects on bacteria', *Science progress,* 86(4), pp. 245-270.

Ho Ahn, J., Hwan Jung, K., Seok Lim, E., Min Kim, S., Ok Han, S. and Um, Y. (2023) 'Recent advances in microbial production of medium chain fatty acid from renewable carbon resources: A comprehensive review', *Bioresource Technology,* 381, pp. 129147. DOI: 10.1016/j.biortech.2023.129147.

Hoerr, F. J. (2010) 'Clinical aspects of immunosuppression in poultry', *Avian diseases,* 54(1), pp. 2-15.

Højberg, O., Canibe, N., Poulsen, H. D., Hedemann, M. S. and Jensen, B. B. (2005) 'Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets', *Appl Environ Microbiol,* 71(5), pp. 2267-77. DOI: 10.1128/aem.71.5.2267-2277.2005.

Holmes, A. H., Moore, L. S., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P. J. and Piddock, L. J. (2016) 'Understanding the mechanisms and drivers of antimicrobial resistance', *The Lancet,* 387(10014), pp. 176-187.

Hongguang, W. (2023) 'Production of the Livestock Sector: A 60-Year Review and Outlook', *China's Food Security: Strategies and Countermeasures*. Singapore: Springer Nature Singapore, pp. 135-163.

Hood, J. R., Wilkinson, J. M. and Cavanagh, H. M. A. (2003) 'Evaluation of Common Antibacterial Screening Methods Utilized in Essential Oil Research', *Journal of Essential Oil Research,* 15(6), pp. 428-433. DOI: 10.1080/10412905.2003.9698631.

Horn, N., Miller, G., Ajuwon, K. M. and Adeola, O. (2017) 'Ability of garlic-derived diallyl disulfide and diallyl trisulfide supplemented by oral gavage to mitigate effects of an acute postweaning feed and water deprivation event in nursery pigs1', *Journal of Animal Science,* 95(8), pp. 3579-3590. DOI: 10.2527/jas.2017.1545.

Hu, Q., Zhou, M. and wei, S. (2018) 'Progress on the Antimicrobial Activity Research of Clove Oil and Eugenol in the Food Antisepsis Field', *Journal of Food Science,* 83(6), pp. 1476-1483. DOI: 10.1111/1750-3841.14180.

Huang, Q., Liu, X., Zhao, G., Hu, T. and Wang, Y. (2018) 'Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production', *Animal Nutrition,* 4(2), pp. 137-150. DOI: 10.1016/j.aninu.2017.09.004.

Hui, X., Wu, G., Han, D., Gong, X., Stipkovits, L., Wu, X., Tang, S., Brennan, M. A. and Brennan, C. S. (2021) 'Bioactive compounds from blueberry and blackcurrant powder alter the physicochemical and hypoglycaemic properties of oat bran paste', *LWT,* 143, pp. 111167.

Hyldgaard, M., Mygind, T. and Meyer, R. L. (2012) 'Essential Oils in Food Preservation: Mode of Action, Synergies, and Interactions with Food Matrix Components', *Frontiers in Microbiology,* 3. DOI: 10.3389/fmicb.2012.00012.

Ichikawa, M., Ide, N. and Ono, K. (2006) 'Changes in Organosulfur Compounds in Garlic Cloves during Storage', *Journal of Agricultural and Food Chemistry,* 54(13), pp. 4849-4854. DOI: 10.1021/jf060083o.

Immerseel, F. V., Buck, J. D., Pasmans, F., Huyghebaert, G., Haesebrouck, F. and Ducatelle, R. (2004) 'Clostridium perfringens in poultry: an emerging threat for animal and public health', *Avian Pathology,* 33(6), pp. 537-549. DOI: 10.1080/03079450400013162.

Iqbal, Y., Cottrell, J. J., Suleria, H. A. R. and Dunshea, F. R. (2020) 'Gut Microbiota-Polyphenol Interactions in Chicken: A Review', *Animals,* 10(8), pp. 1391. Available at: https://www.mdpi.com/2076-2615/10/8/1391.

Jackman, J. A., Boyd, R. D. and Elrod, C. C. (2020) 'Medium-chain fatty acids and monoglycerides as feed additives for pig production: towards gut health improvement and feed pathogen mitigation', *Journal of Animal Science and Biotechnology,* 11(1), pp. 44. DOI: 10.1186/s40104-020-00446-1.

Jang, C., Hui, S., Lu, W., Cowan, A. J., Morscher, R. J., Lee, G., Liu, W., Tesz, G. J., Birnbaum, M. J. and Rabinowitz, J. D. (2018) 'The small intestine converts dietary fructose into glucose and organic acids', *Cell metabolism,* 27(2), pp. 351-361. e3.

Jeong, C.-H., Jang, C.-W., Lee, K.-Y., Kim, I.-H. and Shim, K.-H. (2012) 'Chemical components and anti-oxidant activities of black currant', *Korean Journal of Food Preservation,* 19(2), pp. 263-270.

Ji, Q.-Y., Wang, W., Yan, H., Qu, H., Liu, Y., Qian, Y. and Gu, R. (2023) 'The Effect of Different Organic Acids and Their Combination on the Cell Barrier and Biofilm of Escherichia coli', *Foods,* 12(16), pp. 3011.

Jiang, B., Fang, X., Fu, D., Wu, W., Han, Y., Chen, H., Liu, R. and Gao, H. (2022) 'Exogenous salicylic acid regulates organic acids metabolism in postharvest blueberry fruit', *Frontiers in Plant Science,* 13, pp. 1024909.

Jiménez, M. J., Berrios, R., Stelzhammer, S. and Bracarense, A. (2020) 'Ingestion of organic acids and cinnamaldehyde improves tissue homeostasis of piglets exposed to enterotoxic Escherichia coli (ETEC)', *J Anim Sci,* 98(2). DOI: 10.1093/jas/skaa012.

Jin, X., Huang, G., Luo, Z., Hu, Y. and Liu, D. (2022) 'Oregano (Origanum vulgare L.) Essential Oil Feed Supplement Protected Broilers Chickens against Clostridium perfringens Induced Necrotic Enteritis', *Agriculture,* 12(1), pp. 18. Available at: https://www.mdpi.com/2077-0472/12/1/18.

Joffré, E., Zurita, J., Calderon Toledo, C. and Gutiérrez-Cortez, S. (2023) 'Recent Progress on Enterotoxigenic E. coli (ETEC) and Antibiotic Resistance in Pathogenic E. coli', in Torres, A.G. (ed.) *Trending Topics in Escherichia coli Research: The Latin American Perspective*. Cham: Springer International Publishing, pp. 33-53.

Kabara, J. J., Swieczkowski, D. M., Conley, A. J. and Truant, J. P. (1972) 'Fatty Acids and Derivatives as Antimicrobial Agents', *Antimicrobial Agents and Chemotherapy,* 2(1), pp. 23-28. DOI: 10.1128/aac.2.1.23.

Kauser, S., Murtaza, M. A., Hussain, A., Imran, M., Kabir, K., Najam, A., An, Q. U., Akram, S., Fatima, H., Batool, S. A., Shehzad, A. and Yaqub, S. (2024) 'Apple pomace, a bioresource of functional and nutritional components with potential of utilization in different food formulations: A review', *Food Chemistry Advances,* 4, pp. 100598. DOI: 10.1016/j.focha.2023.100598.

Kemperman, R. A., Bolca, S., Roger, L. C. and Vaughan, E. E. (2010) 'Novel approaches for analysing gut microbes and dietary polyphenols: challenges and opportunities', *Microbiology,* 156(11), pp. 3224-3231.

Khoramnia, A., Ebrahimpour, A., Ghanbari, R., Ajdari, Z. and Lai, O. M. (2013) 'Improvement of medium chain fatty acid content and antimicrobial activity of coconut oil via solid-state fermentation using a Malaysian Geotrichum candidum', *Biomed Res Int,* 2013, pp. 954542. DOI: 10.1155/2013/954542.

Kim, J. W., Kim, Y. S. and Kyung, K. H. (2004) 'Inhibitory Activity of Essential Oils of Garlic and Onion against Bacteria and Yeasts', *Journal of Food Protection,* 67(3), pp. 499-504. DOI: 10.4315/0362-028X-67.3.499.

Kim, K., Song, M., Liu, Y. and Ji, P. (2022) 'Enterotoxigenic Escherichia coli infection of weaned pigs: Intestinal challenges and nutritional intervention to enhance disease resistance', *Frontiers in Immunology,* 13. DOI: 10.3389/fimmu.2022.885253.

Kim, S. and Rhee, M. (2013) 'Marked synergistic bactericidal effects and mode of action of medium-chain fatty acids in combination with organic acids against Escherichia coli O157: H7', *Applied and environmental microbiology,* 79(21), pp. 6552-6560.

Kinderlerer, J. L. (1994) 'Degradation of the lauric acid oils', *International biodeterioration & biodegradation,* 33(4), pp. 345-354.

Knudsen, K. B. (2019) '6: Nutritional modulation to improve health and welfare', *Poultry and pig nutrition: Challenges of the 21st century*. Wageningen Academic Publishers, pp. 87-96.

Kuete, J. R. N. and Kuete, V. (2023) 'Chapter Three - Harvesting and processing medicinal plants for antibacterial testing', in Kuete, V. (ed.) *Advances in Botanical Research*: Academic Press, pp. 47-60.
Kumar, A., Kheravii, S. K., Ionescu, C., Blanchard, A., Barekatain, R., Bajagai, Y. S. and Wu, S. B. (2021) 'A Microencapsulated Mixture of Eugenol and Garlic Tincture Supplementation Mitigates the Effect of Necrotic Enteritis on Intestinal Integrity and Increases Goblet Cells in Broilers', *Microorganisms,* 9(7). DOI: 10.3390/microorganisms9071451.
Kumar, A., Sharma, N. K., Kheravii, S. K., Keerqin, C., Ionescu, C., Blanchard, A. and Wu, S. B. (2022) 'Potential of a mixture of eugenol and garlic tincture to improve performance and intestinal health in broilers under necrotic enteritis challenge', *Anim Nutr,* 8(1), pp. 26-37. DOI: 10.1016/j.aninu.2021.07.007.
Lacey, J. A., Keyburn, A. L., Ford, M. E., Portela, R. W., Johanesen, P. A., Lyras, D. and Moore, R. J. (2017) 'Conjugation-Mediated Horizontal Gene Transfer of Clostridium perfringens Plasmids in the Chicken Gastrointestinal Tract Results in the Formation of New Virulent Strains', *Appl Environ Microbiol,* 83(24). DOI: 10.1128/aem.01814-17.
Lahiri, D., Nag, M., Dutta, B., Dash, S., Ghosh, S. and Ray, R. R. 'Synergistic Effect of Quercetin with Allicin from the Ethanolic Extract of Allium cepa as a Potent AntiQuorum Sensing and Anti-Biofilm Agent Against Oral Biofilm'. *Advances in Bioprocess Engineering and Technology*. Singapore: Springer Singapore, 69-81.
Lallès, J. P., Bosi, P., Janczyk, P., Koopmans, S. and Torrallardona, D. (2009) 'Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: a review', *Animal,* 3(12), pp. 1625-1643.
Lallès, J.-P., Boudry, G., Favier, C., Le Floc'h, N., Luron, I., Montagne, L., Oswald, I. P., Pié, S., Piel, C. and Sève, B. (2004) 'Gut function and dysfunction in young pigs: physiology', *Animal Research,* 53(4), pp. 301-316.
Langeveld, W. T., Veldhuizen, E. J. A. and Burt, S. A. (2014) 'Synergy between essential oil components and antibiotics: a review', *Critical Reviews in Microbiology,* 40(1), pp. 76-94. DOI: 10.3109/1040841X.2013.763219.
Lanzotti, V., Scala, F. and Bonanomi, G. (2014) 'Compounds from Allium species with cytotoxic and antimicrobial activity', *Phytochemistry Reviews,* 13, pp. 769-791.
Lauridsen, C., Højberg, O. and Canibe, N. (2022) 'Improving gut function in pigs to prevent dysbiosis and post-weaning diarrhoea'.
Lawlor, P. G., Lynch, P. B., Caffrey, P. J., O'Reilly, J. J. and O'Connell, M. K. (2005) 'Measurements of the acid-binding capacity of ingredients used in pig diets', *Irish Veterinary Journal,* 58(8), pp. 447. DOI: 10.1186/2046-0481-58-8-447.
Lawlor, P., Gardiner, G. and Goodband, R. (2020) 'Feeding the weaned piglet', *The suckling and weaned piglet*: Wageningen Academic Publishers, pp. 1304-1309.
Lawson, L. D. and Gardner, C. D. (2005) 'Composition, Stability, and Bioavailability of Garlic Products Used in a Clinical Trial', *Journal of Agricultural and Food Chemistry,* 53(16), pp. 6254-6261. DOI: 10.1021/jf050536+.
Lawson, L. D. and Hunsaker, S. M. (2018) 'Allicin Bioavailability and Bioequivalence from Garlic Supplements and Garlic Foods', *Nutrients,* 10(7). DOI: 10.3390/nu10070812.
Leber, A. L. (2020) *Clinical Microbiology Procedures Handbook.* Wiley.
Lee, K. W. and Lillehoj, H. S. (2021) 'Role of Clostridium perfringens Necrotic Enteritis B-like Toxin in Disease Pathogenesis', *Vaccines (Basel),* 10(1). DOI: 10.3390/vaccines10010061.
Leeb, C., Hegelund, L., Edwards, S., Mejer, H., Roepstorff, A., Rousing, T., Sundrum, A. and Bonde, M. (2014) 'Animal health, welfare and production problems in organic weaner pigs', *Organic Agriculture,* 4(2), pp. 123-133. DOI: 10.1007/s13165-013-0054-y.
Lei, X. J., Park, J. W., Baek, D. H., Kim, J. K. and Kim, I. H. (2017) 'Feeding the blend of organic acids and medium chain fatty acids reduces the diarrhea in piglets orally challenged with enterotoxigenic Escherichia coli K88', *Animal Feed Science and Technology,* 224, pp. 46-51. DOI: 10.1016/j.anifeedsci.2016.11.016.
Lencova, S., Zdenkova, K., Demnerova, K. and Stiborova, H. (2022) 'Short communication: Antibacterial and antibiofilm effect of natural substances and their mixtures over Listeria monocytogenes, Staphylococcus aureus and Escherichia coli', *LWT,* 154, pp. 112777. DOI: 10.1016/j.lwt.2021.112777.
Lengsfeld, C., Deters, A., Faller, G. and Hensel, A. (2004) 'High molecular weight polysaccharides from black currant seeds inhibit adhesion of Helicobacter pylori to human gastric mucosa', *Planta medica,* 70(07), pp. 620-626.
Li, Z., Jiang, B., Zhang, X., Yang, Y., Hardwidge, P. R., Ren, W. and Zhu, G. (2020) 'The role of bacterial cell envelope structures in acid stress resistance in E. coli', *Applied Microbiology and Biotechnology,* 104(7), pp. 2911-2921. DOI: 10.1007/s00253-020-10453-x.
Lillehoj, H., Liu, Y., Calsamiglia, S., Fernandez-Miyakawa, M. E., Chi, F., Cravens, R. L., Oh, S. and Gay, C. G. (2018) 'Phytochemicals as antibiotic alternatives to promote growth and enhance host health', *Vet Res,* 49(1), pp. 76. DOI: 10.1186/s13567-018-0562-6.
Lindgren, K., Bochicchio, D., Hegelund, L., Leeb, C., Mejer, H., Roepstorff, A. and Sundrum, A. (2014) 'Animal health and welfare in production systems for organic fattening pigs', *Organic Agriculture,* 4(2), pp. 135-147. DOI: 10.1007/s13165-014-0069-z.

Ling, K.-H., Wan, M. L. Y., El-Nezami, H. and Wang, M. (2016) 'Protective capacity of resveratrol, a natural polyphenolic compound, against deoxynivalenol-induced intestinal barrier dysfunction and bacterial translocation', *Chemical Research in Toxicology,* 29(5), pp. 823-833.

Liu, F., Koval, M., Ranganathan, S., Fanayan, S., Hancock, W. S., Lundberg, E. K., Beavis, R. C., Lane, L., Duek, P. and McQuade, L. (2016) 'Systems proteomics view of the endogenous human claudin protein family', *Journal of proteome research,* 15(2), pp. 339-359.

Liu, Y., Song, M., Che, T. M., Almeida, J. A., Lee, J. J., Bravo, D., Maddox, C. W. and Pettigrew, J. E. (2013) 'Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli', *J Anim Sci,* 91(11), pp. 5294-306. DOI: 10.2527/jas.2012-6194.

Liu, Y., Song, M., Che, T., Bravo, D., Maddox, C. and Pettigrew, J. (2014) 'Effects of capsicum oleoresin, garlic botanical, and turmeric oleoresin on gene expression profile of ileal mucosa in weaned pigs', *Journal of Animal Science,* 92(8), pp. 3426-3440.

Lu, X., Rasco, B. A., Jabal, J. M. F., Aston, D. E., Lin, M. and Konkel, M. E. (2011) 'Investigating Antibacterial Effects of Garlic (Allium sativum) Concentrate and Garlic-Derived Organosulfur Compounds on Campylobacter jejuni by Using Fourier Transform Infrared Spectroscopy, Raman Spectroscopy, and Electron Microscopy', *Applied and Environmental Microbiology,* 77(15), pp. 5257-5269. DOI: 10.1128/AEM.02845-10.

Luise, D., Correa, F., Bosi, P. and Trevisi, P. (2020) 'A Review of the Effect of Formic Acid and Its Salts on the Gastrointestinal Microbiota and Performance of Pigs', *Animals,* 10(5), pp. 887. Available at: https://www.mdpi.com/2076-2615/10/5/887.

Luise, D., Lauridsen, C., Bosi, P. and Trevisi, P. (2019a) 'Methodology and application of Escherichia coli F4 and F18 encoding infection models in post-weaning pigs', *Journal of Animal Science and Biotechnology,* 10(1), pp. 53. DOI: 10.1186/s40104-019-0352-7.

Luise, D., Lauridsen, C., Bosi, P. and Trevisi, P. (2019b) 'Methodology and application of Escherichia coli F4 and F18 encoding infection models in post-weaning pigs', *Journal of Animal Science and Biotechnology,* 10. DOI: 10.1186/s40104-019-0352-7.

Luppi, A., Gibellini, M., Gin, T., Vangroenweghe, F., Vandenbroucke, V., Bauerfeind, R., Bonilauri, P., Labarque, G. and Hidalgo, Á. (2016) 'Prevalence of virulence factors in enterotoxigenic Escherichia coli isolated from pigs with post-weaning diarrhoea in Europe', *Porcine Health Management,* 2(1), pp. 20. DOI: 10.1186/s40813-016-0039-9.

Lyu, F., Luiz, S. F., Azeredo, D. R. P., Cruz, A. G., Ajlouni, S. and Ranadheera, C. S. (2020) 'Apple Pomace as a Functional and Healthy Ingredient in Food Products: A Review', *Processes,* 8(3), pp. 319. Available at: https://www.mdpi.com/2227-9717/8/3/319.

Ma, A. and Qi, X. (2021) 'Mining plant metabolomes: Methods, applications, and perspectives', *Plant Commun,* 2(5), pp. 100238. DOI: 10.1016/j.xplc.2021.100238.

Ma, J., Piao, X., Mahfuz, S., Long, S. and Wang, J. (2022) 'The interaction among gut microbes, the intestinal barrier and short chain fatty acids', *Animal Nutrition,* 9, pp. 159-174.

Magryś, A., Olender, A. and Tchórzewska, D. (2021) 'Antibacterial properties of Allium sativum L. against the most emerging multidrug-resistant bacteria and its synergy with antibiotics', *Archives of microbiology,* 203(5), pp. 2257-2268.

Marchese, A., Barbieri, R., Sanches-Silva, A., Daglia, M., Nabavi, S. F., Jafari, N. J., Izadi, M., Ajami, M. and Nabavi, S. M. (2016) 'Antifungal and antibacterial activities of allicin: A review', *Trends in Food Science & Technology,* 52, pp. 49-56.

Marks, H., Grześkowiak, Ł., Martinez-Vallespin, B., Dietz, H. and Zentek, J. (2022) 'Porcine and Chicken Intestinal Epithelial Cell Models for Screening Phytogenic Feed Additives-Chances and Limitations in Use as Alternatives to Feeding Trials', *Microorganisms,* 10(3). DOI: 10.3390/microorganisms10030629.

Martău, G. A., Teleky, B.-E., Ranga, F., Pop, I. D. and Vodnar, D. C. (2021) 'Apple Pomace as a Sustainable Substrate in Sourdough Fermentation', *Frontiers in Microbiology,* 12. DOI: 10.3389/fmicb.2021.742020.

Martins, N., Petropoulos, S. and Ferreira, I. C. F. R. (2016) 'Chemical composition and bioactive compounds of garlic (Allium sativum L.) as affected by pre- and post-harvest conditions: A review', *Food Chemistry,* 211, pp. 41-50. DOI: 10.1016/j.foodchem.2016.05.029.

Massacci, F. R., Tofani, S., Forte, C., Bertocchi, M., Lovito, C., Orsini, S., Tentellini, M., Marchi, L., Lemonnier, G., Luise, D., Blanc, F., Castinel, A., Bevilacqua, C., Rogel-Gaillard, C., Pezzotti, G., Estellé, J., Trevisi, P. and Magistrali, C. F. (2020) 'Host genotype and amoxicillin administration affect the incidence of diarrhoea and faecal microbiota of weaned piglets during a natural multiresistant ETEC infection', *Journal of Animal Breeding and Genetics,* 137(1), pp. 60-72. DOI: 10.1111/jbg.12432.

Matsue, M., Mori, Y., Nagase, S., Sugiyama, Y., Hirano, R., Ogai, K., Ogura, K., Kurihara, S. and Okamoto, S. (2019) 'Measuring the Antimicrobial Activity of Lauric Acid against Various Bacteria in Human Gut Microbiota Using a New Method', *Cell Transplant,* 28(12), pp. 1528-1541. DOI: 10.1177/0963689719881366.

Maurer, L. M., Yohannes, E., Bondurant, S. S., Radmacher, M. and Slonczewski, J. L. (2005) 'pH regulates genes for flagellar motility, catabolism, and oxidative stress in Escherichia coli K-12', *Journal of bacteriology,* 187(1), pp. 304-319.

McMurdie, P. J. and Holmes, S. (2013) 'phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data', *PloS one,* 8(4), pp. e61217.

Méndez Lagunas, L. L. and Castaigne, F. (2008) 'Effect of temperature cycling on allinase activity in garlic', *Food Chemistry,* 111(1), pp. 56-60. DOI: 10.1016/j.foodchem.2008.03.035.

Mesa-Pineda, C., Navarro-Ruíz, J. L., López-Osorio, S., Chaparro-Gutiérrez, J. J. and Gómez-Osorio, L. M. (2021) 'Chicken Coccidiosis: From the Parasite Lifecycle to Control of the Disease', *Frontiers in Veterinary Science,* 8. DOI: 10.3389/fvets.2021.787653.

Miladinović, B., Kostić, M., Šavikin, K., Đorđević, B., Mihajilov-Krstev, T., Živanović, S. and Kitić, D. (2014) 'Chemical profile and antioxidative and antimicrobial activity of juices and extracts of 4 black currants varieties (Ribes nigrum L.)', *J Food Sci,* 79(3), pp. C301-9. DOI: 10.1111/1750-3841.12364.

Miron, T., Rabinkov, A., Mirelman, D., Wilchek, M. and Weiner, L. (2000) 'The mode of action of allicin: its ready permeability through phospholipid membranes may contribute to its biological activity', *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 1463(1), pp. 20-30. DOI: 10.1016/S0005-2736(99)00174-1.

Moco, S. (2022) 'Studying Metabolism by NMR-Based Metabolomics', *Frontiers in Molecular Biosciences,* 9. DOI: 10.3389/fmolb.2022.882487.

Mofasser Hossain, A. K. M., Brennan, M. A., Mason, S. L., Guo, X. and Brennan, C. S. (2017) 'The Combined Effect of Blackcurrant Powder and Wholemeal Flours to Improve Health Promoting Properties of Cookies', *Plant Foods for Human Nutrition,* 72(3), pp. 280-287. DOI: 10.1007/s11130-017-0619-0.

Montoya, C., Cochard, B., Flori, A., Cros, D., Lopes, R., Cuellar, T., Espeout, S., Syaputra, I., Villeneuve, P., Pina, M., Ritter, E., Leroy, T. and Billotte, N. (2014) 'Genetic Architecture of Palm Oil Fatty Acid Composition in Cultivated Oil Palm (Elaeis guineensis Jacq.) Compared to Its Wild Relative E. oleifera (H.B.K) Cortés', *PLOS ONE,* 9(5), pp. e95412. DOI: 10.1371/journal.pone.0095412.

Moore, J. P., Li, H., Engmann, M. L., Bischof, K. M., Kunka, K. S., Harris, M. E., Tancredi, A. C., Ditmars, F. S., Basting, P. J., George, N. S., Bhagwat, A. A. and Slonczewski, J. L. (2019) 'Inverted Regulation of Multidrug Efflux Pumps, Acid Resistance, and Porins in Benzoate-Evolved Escherichia coli K-12', *Applied and Environmental Microbiology,* 85(16), pp. e00966-19. DOI: 10.1128/AEM.00966-19.

Moore, R. J. (2016) 'Necrotic enteritis predisposing factors in broiler chickens', *Avian Pathology,* 45(3), pp. 275-281. DOI: 10.1080/03079457.2016.1150587.

Moore, R. J. (2024) 'Necrotic enteritis and antibiotic-free production of broiler chickens: Challenges in testing and using alternative products', *Animal Nutrition,* 16, pp. 288-298. DOI: 10.1016/j.aninu.2023.08.012.

Moran, E. T. (2014) 'Intestinal events and nutritional dynamics predispose Clostridium perfringens virulence in broilers1 1Presented in part at the Inspireform 2014, Pitlochry, Scotland', *Poultry Science,* 93(12), pp. 3028-3036. DOI: 10.3382/ps.2014-04313.

Morgan, N. K. (2017) 'Managing gut health without reliance on antimicrobials in poultry', *Animal Production Science,* 57(11), pp. 2270-2279. DOI: 10.1071/an17288.

M'Sadeq, S. A., Wu, S., Swick, R. A. and Choct, M. (2015) 'Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide', *Animal Nutrition,* 1(1), pp. 1-11. DOI: 10.1016/j.aninu.2015.02.004.

Müller, A., Eller, J., Albrecht, F., Prochnow, P., Kuhlmann, K., Bandow, J. E., Slusarenko, A. J. and Leichert, L. I. (2016) 'Allicin Induces Thiol Stress in Bacteria through S-Allylmercapto Modification of Protein Cysteines', *J Biol Chem,* 291(22), pp. 11477-90. DOI: 10.1074/jbc.M115.702308.

Naganawa, R., Iwata, N., Ishikawa, K., Fukuda, H., Fujino, T. and Suzuki, A. (1996) 'Inhibition of microbial growth by ajoene, a sulfur-containing compound derived from garlic', *Applied and environmental microbiology,* 62(11), pp. 4238-4242.

National Center for Biotechnology Information (2024). PubChem Compound Summary for CID 65036, Allicin. Retrieved May 1, 2024 from https://pubchem.ncbi.nlm.nih.gov/compound/Allicin.

Nayak, P. K., Chandrasekar, C. M., Sundarsingh, A. and Kesavan, R. K. (2020) 'Effect of in-vitro digestion on the bio active compounds and biological activities of fruit pomaces', *Journal of Food Science and Technology,* 57(12), pp. 4707-4715. DOI: 10.1007/s13197-020-04507-1.

Nguyen, D. H., Seok, W. J. and Kim, I. H. (2020) 'Organic Acids Mixture as a Dietary Additive for Pigs-A Review', *Animals (Basel),* 10(6). DOI: 10.3390/ani10060952.

Nguyen, U. V., Coddens, A., Melkebeek, V., Devriendt, B., Goetstouwers, T., Poucke, M. V., Peelman, L. and Cox, E. (2017) 'High susceptibility prevalence for F4+ and F18+ Escherichia coli in Flemish pigs', *Veterinary Microbiology,* 202, pp. 52-57. DOI: 10.1016/j.vetmic.2016.01.014.

Nikolic, I., Vukovic, D., Gavric, D., Cvetanovic, J., Aleksic Sabo, V., Gostimirovic, S., Narancic, J. and Knezevic, P. (2022) 'An Optimized Checkerboard Method for Phage-Antibiotic Synergy Detection', *Viruses,* 14(7). DOI: 10.3390/v14071542.

Nohynek, L. J., Alakomi, H.-L., Kähkönen, M. P., Heinonen, M., Helander, I. M., Oksman-Caldentey, K.-M. and Puupponen-Pimiä, R. H. (2006) 'Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens', *Nutrition and cancer,* 54(1), pp. 18-32.

Nour, V., Trandafir, I. and Ionica, M. E. (2011) 'Ascorbic acid, anthocyanins, organic acids and mineral content of some black and red currant cultivars', *Fruits,* 66(5), pp. 353-362.

O'Neill, L., Manzanilla, E. G., Ekhlas, D. and Leonard, F. C. (2023) 'Antimicrobial Resistance in Commensal Escherichia coli of the Porcine Gastrointestinal Tract', *Antibiotics,* 12(11), pp. 1616. Available at: https://www.mdpi.com/2079-6382/12/11/1616.

O'Toole, G. A. (2024) 'We have a community problem', *Journal of Bacteriology,* 206(4), pp. e00073-24. DOI: 10.1128/jb.00073-24.

O-Thong, S., Zhu, X., Angelidaki, I., Zhang, S. and Luo, G. (2020) 'Chapter Two - Medium chain fatty acids production by microbial chain elongation: Recent advances', in Li, Y. & Khanal, S.K. (eds.) *Advances in Bioenergy*. Elsevier, pp. 63-99.

Pakbin, B., Brück, W. M. and Rossen, J. W. A. (2021) 'Virulence Factors of Enteric Pathogenic Escherichia coli: A Review', *International Journal of Molecular Sciences,* 22(18), pp. 9922. Available at: https://www.mdpi.com/1422-0067/22/18/9922.

Pandey, A. K., Kumar, P. and Saxena, M. J. (2019) 'Feed Additives in Animal Health', in Gupta, R.C., Srivastava, A. & Lall, R. (eds.) *Nutraceuticals in Veterinary Medicine*. Cham: Springer International Publishing, pp. 345-362.

Papuc, C., Goran, G. V., Predescu, C. N., Nicorescu, V. and Stefan, G. (2017) 'Plant Polyphenols as Antioxidant and Antibacterial Agents for Shelf-Life Extension of Meat and Meat Products: Classification, Structures, Sources, and Action Mechanisms', *Comprehensive Reviews in Food Science and Food Safety,* 16(6), pp. 1243-1268. DOI: 10.1111/1541-4337.12298.

Park, S., Lee, J. W., Cowieson, A. J., Pappenberger, G. and Woyengo, T. A. (2020) 'Soybean meal allergenic protein degradation and gut health of piglets fed protease-supplemented diets', *J Anim Sci,* 98(10). DOI: 10.1093/jas/skaa308.

Patience, J. F. and Ramirez, A. (2022) 'Invited review: strategic adoption of antibiotic-free pork production: the importance of a holistic approach', *Translational Animal Science,* 6(3), pp. txac063.

Patil, Y., Gooneratne, R. and Ju, X.-H. (2020) 'Interactions between host and gut microbiota in domestic pigs: a review', *Gut Microbes,* 11(3), pp. 310-334. DOI: 10.1080/19490976.2019.1690363.

Pedersen, K. S., Stege, H. and Nielsen, J. P. (2011) 'Evaluation of a microwave method for dry matter determination in faecal samples from weaned pigs with or without clinical diarrhoea', *Preventive veterinary medicine,* 100(3-4), pp. 163-170.

Pedersen, K., Bjerrum, L., Eske Heuer, O., Lo Fo Wong, D. M. and Nauerby, B. (2008) 'Reproducible infection model for Clostridium perfringens in broiler chickens', *Avian Diseases,* 52(1), pp. 34-39.

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., Pearl, E. J., Petersen, O. H., Rawle, F., Reynolds, P., Rooney, K., Sena, E. S., Silberberg, S. D., Steckler, T. and Würbel, H. (2020) 'The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research', *PLOS Biology,* 18(7), pp. e3000410. DOI: 10.1371/journal.pbio.3000410.

Petropoulos, S., Fernandes, Â., Barros, L., Ciric, A., Sokovic, M. and Ferreira, I. C. F. R. (2018) 'Antimicrobial and antioxidant properties of various Greek garlic genotypes', *Food Chemistry,* 245, pp. 7-12. DOI: 10.1016/j.foodchem.2017.10.078.

Pham, V. H., Abbas, W., Huang, J., He, Q., Zhen, W., Guo, Y. and Wang, Z. (2022) 'Effect of blending encapsulated essential oils and organic acids as an antibiotic growth promoter alternative on growth performance and intestinal health in broilers with necrotic enteritis', *Poult Sci,* 101(1), pp. 101563. DOI: 10.1016/j.psj.2021.101563.

Pham, V. H., Kan, L., Huang, J., Geng, Y., Zhen, W., Guo, Y., Abbas, W. and Wang, Z. (2020) 'Dietary encapsulated essential oils and organic acids mixture improves gut health in broiler chickens challenged with necrotic enteritis', *J Anim Sci Biotechnol,* 11, pp. 18. DOI: 10.1186/s40104-019-0421-y.

Pham, V.H., Kan, L., Huang, J., Bui, X., Se, J., Bui, Q. and Yi, S., 2020. Encapsulated essential oils and organic acids improve gut health and growth performance of broilers under Clostridium perfringens challenge. *Journal of Animal Science and Biotechnology*, 11(1), pp.1-11. DOI:10.1186/s40104-019-0421-y.

Pham, V.H., Kan, L., Huang, J., Bui, X., Se, J., Bui, Q. and Yi, S., 2022. Encapsulated essential oils and organic acids as antibiotic growth promoter alternatives in broiler chickens challenged with necrotic enteritis. *Poultry Science Journal*, 101(1), pp.563-574. DOI:10.1016/j.psj.2021.101563.

Pham, V.H., Kan, L., Huang, J., Bui, X., Se, J., Bui, Q. and Yi, S., 2023. Dietary coated essential oils and organic acids enhance growth performance and gut health of broilers infected with avian pathogenic Escherichia coli. *Animal Nutrition*, 9, pp.10-21. 10.1016/j.aninu.2022.09.010.

Phan, A. D. T., Netzel, G., Chhim, P., Netzel, M. E. and Sultanbawa, Y. (2019) 'Phytochemical Characteristics and Antimicrobial Activity of Australian Grown Garlic (Allium Sativum L.) Cultivars', *Foods,* 8(9), pp. 358. Available at: https://www.mdpi.com/2304-8158/8/9/358.

Pieszka, M., Szczurek, P., Bederska-Łojewska, D., Migdał, W., Pieszka, M., Gogol, P. and Jagusiak, W. (2017) 'The effect of dietary supplementation with dried fruit and vegetable pomaces on production parameters and meat quality in fattening pigs', *Meat Science,* 126, pp. 1-10. DOI: 10.1016/j.meatsci.2016.11.016.

Pluske, J. R., Turpin, D. L. and Kim, J.-C. (2018) 'Gastrointestinal tract (gut) health in the young pig', *Animal Nutrition,* 4(2), pp. 187-196. DOI: 10.1016/j.aninu.2017.12.004.

Pomothy, J. M., Barna, R. F., Pászti, E. A., Babiczky, Á., Szóládi, Á., Jerzsele, Á. and Gere, E. P. (2020) 'Beneficial effects of rosmarinic acid on IPEC-J2 cells exposed to the combination of deoxynivalenol and T-2 toxin', *Mediators of Inflammation,* 2020.

Pott, D. M., Durán-Soria, S., Allwood, J. W., Pont, S., Gordon, S. L., Jennings, N., Austin, C., Stewart, D., Brennan, R. M., Masny, A., Sønsteby, A., Krüger, E., Jarret, D., Vallarino, J. G., Usadel, B. and Osorio, S. (2023) 'Dissecting the impact of environment, season and genotype on blackcurrant fruit quality traits', *Food Chemistry,* 402, pp. 134360. DOI: 10.1016/j.foodchem.2022.134360.

Prescott, J. F., Parreira, V. R., Mehdizadeh Gohari, I., Lepp, D. and Gong, J. (2016a) 'The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know: a review', *Avian Pathology,* 45(3), pp. 288-294. DOI: 10.1080/03079457.2016.1139688.

Prescott, J. F., Smyth, J. A., Shojadoost, B. and Vince, A. (2016b) 'Experimental reproduction of necrotic enteritis in chickens: a review', *Avian Pathology,* 45(3), pp. 317-322. DOI: 10.1080/03079457.2016.1141345.

Puupponen-Pimiä, R., Nohynek, L., Alakomi, H.-L. and Oksman-Caldentey, K.-M. (2005) 'Bioactive berry compounds—novel tools against human pathogens', *Applied Microbiology and Biotechnology,* 67(1), pp. 8-18. DOI: 10.1007/s00253-004-1817-x.

Puupponen-Pimiä, R., Nohynek, L., Hartmann-Schmidlin, S., Kähkönen, M., Heinonen, M., Määttä-Riihinen, K. and Oksman-Caldentey, K. M. (2005) 'Berry phenolics selectively inhibit the growth of intestinal pathogens', *Journal of Applied Microbiology,* 98(4), pp. 991-1000. DOI: 10.1111/j.1365-2672.2005.02547.x.

Puupponen-Pimiä, R., Nohynek, L., Meier, C., Kähkönen, M., Heinonen, M., Hopia, A. and Oksman-Caldentey, K.-M. (2001) 'Antimicrobial properties of phenolic compounds from berries', *Journal of Applied Microbiology,* 90(4), pp. 494-507. DOI: 10.1046/j.1365-2672.2001.01271.x.

Queji, M. D., Wosiacki, G., Cordeiro, G. A., Peralta-Zamora, P. G. and Nagata, N. (2010) 'Determination of simple sugars, malic acid and total phenolic compounds in apple pomace by infrared spectroscopy and PLSR', *International journal of food science & technology,* 45(3), pp. 602-609.

Rabetafika, H. N., Bchir, B., Blecker, C. and Richel, A. (2014) 'Fractionation of apple by-products as source of new ingredients: Current situation and perspectives', *Trends in Food Science & Technology,* 40(1), pp. 99-114. DOI: 10.1016/j.tifs.2014.08.004.

Rana, S., Gupta, S., Rana, A. and Bhushan, S. (2015) 'Functional properties, phenolic constituents and antioxidant potential of industrial apple pomace for utilization as active food ingredient', *Food Science and Human Wellness,* 4(4), pp. 180-187. DOI: 10.1016/j.fshw.2015.10.001.

Raudsepp, P., Koskar, J., Anton, D., Meremäe, K., Kapp, K., Laurson, P., Bleive, U., Kaldmäe, H., Roasto, M. and Püssa, T. (2019) 'Antibacterial and antioxidative properties of different parts of garden rhubarb, blackcurrant, chokeberry and blue honeysuckle', *Journal of the Science of Food and Agriculture,* 99(5), pp. 2311-2320. DOI: 10.1002/jsfa.9429.

Ravindran, V. and Kornegay, E. T. (1993) 'Acidification of weaner pig diets: A review', *Journal of the Science of Food and Agriculture,* 62(4), pp. 313-322. DOI: 10.1002/jsfa.2740620402.

Reis, J. H., Gebert, R. R., Barreta, M., Baldissera, M. D., Dos Santos, I. D., Wagner, R., Campigotto, G., Jaguezeski, A. M., Gris, A. and de Lima, J. L. (2018) 'Effects of phytogenic feed additive based on thymol, carvacrol and cinnamic aldehyde on body weight, blood parameters and environmental bacteria in broilers chickens', *Microbial pathogenesis,* 125, pp. 168-176.

Reißner, A.-M., Al-Hamimi, S., Quiles, A., Schmidt, C., Struck, S., Hernando, I., Turner, C. and Rohm, H. (2019) 'Composition and physicochemical properties of dried berry pomace', *Journal of the Science of Food and Agriculture,* 99(3), pp. 1284-1293. DOI: 10.1002/jsfa.9302.

Reiter, J., Hübbers, A. M., Albrecht, F., Leichert, L. I. O. and Slusarenko, A. J. (2020) 'Allicin, a natural antimicrobial defence substance from garlic, inhibits DNA gyrase activity in bacteria', *International Journal of Medical Microbiology,* 310(1), pp. 151359. DOI: 10.1016/j.ijmm.2019.151359.

Reiter, J., Levina, N., Van der Linden, M., Gruhlke, M., Martin, C. and Slusarenko, A. J. (2017) 'Diallylthiosulfinate (Allicin), a Volatile Antimicrobial from Garlic (Allium sativum), Kills Human Lung Pathogenic Bacteria, Including MDR Strains, as a Vapor', *Molecules,* 22(10), pp. 1711. Available at: https://www.mdpi.com/1420-3049/22/10/1711.

Rhouma, M., Fairbrother, J. M., Beaudry, F. and Letellier, A. (2017a) 'Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies', *Acta Veterinaria Scandinavica,* 59(1), pp. 31. DOI: 10.1186/s13028-017-0299-7.

Rhouma, M., Fairbrother, J. M., Beaudry, F. and Letellier, A. (2017b) 'Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies', *Acta Vet Scand,* 59(1), pp. 31. DOI: 10.1186/s13028-017-0299-7.

Rhouma, M., Fairbrother, J. M., Thériault, W., Beaudry, F., Bergeron, N., Laurent-Lewandowski, S. and Letellier, A. (2017c) 'The fecal presence of enterotoxin and F4 genes as an indicator of efficacy of treatment with colistin sulfate in pigs', *BMC Microbiology,* 17(1), pp. 6. DOI: 10.1186/s12866-016-0915-0.

Ripken, D. and Hendriks, H. (2015) *Porcine ex vivo intestinal segment model.* Springer International Publishing.

Rist, V. T., Weiss, E., Eklund, M. and Mosenthin, R. (2013) 'Impact of dietary protein on microbiota composition and activity in the gastrointestinal tract of piglets in relation to gut health: a review', *Animal,* 7(7), pp. 1067-78. DOI: 10.1017/s1751731113000062.

Rivera-Gomis, J., Peres Rubio, C., Martínez Conesa, C., Otal Salaverri, J., Cerón, J. J., Tortosa, D. E. and Cubero Pablo, M. J. (2020) 'Effects of Dietary Supplementation of Garlic and Oregano Essential Oil on Biomarkers of Oxidative Status, Stress and Inflammation in Postweaning Piglets', *Animals,* 10(11), pp. 2093. Available at: https://www.mdpi.com/2076-2615/10/11/2093.

Rivlin, R. S. (2001) 'Historical Perspective on the Use of Garlic', *The Journal of Nutrition,* 131(3), pp. 951S-954S. DOI: 10.1093/jn/131.3.951S.

Rood, J. I., Adams, V., Lacey, J., Lyras, D., McClane, B. A., Melville, S. B., Moore, R. J., Popoff, M. R., Sarker, M. R. and Songer, J. G. (2018) 'Expansion of the Clostridium perfringens toxin-based typing scheme', *Anaerobe,* 53, pp. 5-10.

Ross, L. N. and Woodward, J. F. (2016) 'Koch's postulates: An interventionist perspective', *Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences,* 59, pp. 35-46. DOI: 10.1016/j.shpsc.2016.06.001.

Ross, Z. M., O'Gara, E. A., Hill, D. J., Sleightholme, H. V. and Maslin, D. J. (2001) 'Antimicrobial Properties of Garlic Oil against Human Enteric Bacteria: Evaluation of Methodologies and Comparisons with Garlic Oil Sulfides and Garlic Powder', *Applied and Environmental Microbiology,* 67(1), pp. 475-480. DOI: 10.1128/AEM.67.1.475-480.2001.

Rossi, B., Toschi, A., Piva, A. and Grilli, E. (2020) 'Single components of botanicals and nature-identical compounds as a non-antibiotic strategy to ameliorate health status and improve performance in poultry and pigs', *Nutrition Research Reviews,* 33(2), pp. 218-234. DOI: 10.1017/S0954422420000013.

Rouf, R., Uddin, S. J., Sarker, D. K., Islam, M. T., Ali, E. S., Shilpi, J. A., Nahar, L., Tiralongo, E. and Sarker, S. D. (2020) 'Antiviral potential of garlic (Allium sativum) and its organosulfur compounds: A systematic update of pre-clinical and clinical data', *Trends Food Sci Technol,* 104, pp. 219-234. DOI: 10.1016/j.tifs.2020.08.006.

Rubinskiene, M., Viskelis, P., Jasutiene, I., Viskeliene, R. and Bobinas, C. (2005) 'Impact of various factors on the composition and stability of black currant anthocyanins', *Food Research International,* 38(8), pp. 867-871. DOI: 10.1016/j.foodres.2005.02.027.

Ruiz, R., García, M. P., Lara, A. and Rubio, L. A. (2010) 'Garlic derivatives (PTS and PTS-O) differently affect the ecology of swine faecal microbiota in vitro', *Veterinary Microbiology,* 144(1-2), pp. 110-117.

Russell, J. B. and Diez-Gonzalez, F. (1997) 'The Effects of Fermentation Acids on Bacterial Growth', in Poole, R.K. (ed.) *Advances in Microbial Physiology*. Academic Press, pp. 205-234.

Saco, Y., Peña, R., Matas-Quintanilla, M., Ibáñez-López, F. J., Piñeiro, M., Sotillo, J., Bassols, A. and Gutiérrez, A. M. (2023) 'Influence of the circadian cycle, sex and production stage on the reference values of parameters related to stress and pathology in porcine saliva', *Porcine Health Management,* 9(1), pp. 42. DOI: 10.1186/s40813-023-00337-7.

Saeki, E. K., Kobayashi, R. K. T. and Nakazato, G. (2020) 'Quorum sensing system: Target to control the spread of bacterial infections', *Microbial Pathogenesis,* 142, pp. 104068. DOI: 10.1016/j.micpath.2020.104068.

Salehi, B., Zucca, P., Orhan, I. E., Azzini, E., Adetunji, C. O., Mohammed, S. A., Banerjee, S. K., Sharopov, F., Rigano, D., Sharifi-Rad, J., Armstrong, L., Martorell, M., Sureda, A., Martins, N., Selamoğlu, Z. and Ahmad, Z. (2019) 'Allicin and health: A comprehensive review', *Trends in Food Science & Technology,* 86, pp. 502-516. DOI: 10.1016/j.tifs.2019.03.003.

Sales, J. (2013) 'Effects of Pharmacological Concentrations of Dietary Zinc Oxide on Growth of Post-weaning Pigs: A Meta-analysis', *Biological Trace Element Research,* 152(3), pp. 343-349. DOI: 10.1007/s12011-013-9638-3.

Satam, H., Joshi, K., Mangrolia, U., Waghoo, S., Zaidi, G., Rawool, S., Thakare, R. P., Banday, S., Mishra, A. K. and Das, G. (2023) 'Next-generation sequencing technology: current trends and advancements', *Biology,* 12(7), pp. 997.

Sehm, J., Lindermayer, H., Dummer, C., Treutter, D. and Pfaffl, M. W. (2007) 'The influence of polyphenol rich apple pomace or red-wine pomace diet on the gut morphology in weaning piglets', *Journal of Animal Physiology and Animal Nutrition,* 91(7-8), pp. 289-296. DOI: 10.1111/j.1439-0396.2006.00650.x.

Sehm, J., Lindermayer, H., Meyer, H. H. D. and Pfaffl, M. W. (2006) 'The influence of apple- and red-wine pomace rich diet on mRNA expression of inflammatory and apoptotic markers in different piglet organs', *Animal Science,* 82(6), pp. 877-887. DOI: 10.1017/ASC200699.

Serrano-Jara, D., Rivera-Gomis, J., Tornel, J. A., Bernabé, A., Martínez-Conesa, C., Navarro, J. A., Cánovas, R., Otal, J. and Cubero, M. J. (2023a) 'Effects of dietary supplementation with purple garlic powder and oregano essential oil on intestinal health in post-weaning piglets from commercial farms', *Veterinary Research Communications,* 47(2), pp. 901-909.

Serrano-Jara, D., Rivera-Gomis, J., Tornel, J. A., Jordán, M. J., Martínez-Conesa, C. and Pablo, M. J. C. (2023b) 'Oregano Essential Oil and Purple Garlic Powder Effects on Intestinal Health, Microbiota Indicators and Antimicrobial Resistance as Feed Additives in Weaning Piglets', *Animals,* 14(1), pp. 111.

Shilling, M., Matt, L., Rubin, E., Visitacion, M. P., Haller, N. A., Grey, S. F. and Woolverton, C. J. (2013) 'Antimicrobial effects of virgin coconut oil and its medium-chain fatty acids on Clostridium difficile', *Journal of medicinal food,* 16(12), pp. 1079-1085.

Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S. and Hayashi, H. (2002) 'Complete genome sequence of Clostridium perfringens</i>, an anaerobic flesh-eater', *Proceedings of the National Academy of Sciences,* 99(2), pp. 996-1001. DOI: 10.1073/pnas.022493799.

Shojadoost, B., Vince, A. R. and Prescott, J. F. (2012) 'The successful experimental induction of necrotic enteritis in chickens by Clostridium perfringens: a critical review', *Veterinary Research,* 43(1), pp. 74. DOI: 10.1186/1297-9716-43-74.

Skinner, R. C., Gigliotti, J. C., Ku, K.-M. and Tou, J. C. (2018) 'A comprehensive analysis of the composition, health benefits, and safety of apple pomace', *Nutrition Reviews,* 76(12), pp. 893-909. DOI: 10.1093/nutrit/nuy033.

Skřivanová, E., Marounek, M., Dlouhá, G. and Kaňka, J. (2005) 'Susceptibility of Clostridium perfringens to C2–C18 fatty acids', *Letters in Applied Microbiology,* 41(1), pp. 77-81. DOI: 10.1111/j.1472-765X.2005.01709.x.

Slimestad, R. and Solheim, H. (2002) 'Anthocyanins from Black Currants (Ribes nigrum L.)', *Journal of Agricultural and Food Chemistry,* 50(11), pp. 3228-3231. DOI: 10.1021/jf011581u.

Smith, J., Van Den Broek, F., Martorell, J. C., Hackbarth, H., Ruksenas, O. and Zeller, W. (2007) 'Principles and practice in ethical review of animal experiments across Europe: summary of the report of a FELASA working group on ethical evaluation of animal experiments', *Laboratory Animals,* 41(2), pp. 143-160.

Sommerfeld, V. and Santos, R. R. (2021) 'In vitro assays for evaluating phytate degradation in non-ruminants: chances and limitations', *Journal of the Science of Food and Agriculture,* 101(8), pp. 3117-3122. DOI: 10.1002/jsfa.11020.

Sonnert, N. D., Rosen, C. E., Ghazi, A. R., Franzosa, E. A., Duncan-Lowey, B., González-Hernández, J. A., Huck, J. D., Yang, Y., Dai, Y., Rice, T. A., Nguyen, M. T., Song, D., Cao, Y., Martin, A. L., Bielecka, A. A., Fischer, S., Guan, C., Oh, J., Huttenhower, C., Ring, A. M. and Palm, N. W. (2024) 'A host–microbiota interactome reveals extensive transkingdom connectivity', *Nature,* 628(8006), pp. 171-179. DOI: 10.1038/s41586-024-07162-0.

Soren, N. M. (2012) 'Nutritional Manipulations to Optimize Productivity During Environmental Stresses in Livestock', in Sejian, V., Naqvi, S.M.K., Ezeji, T., Lakritz, J. & Lal, R. (eds.) *Environmental Stress and Amelioration in Livestock Production*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 181-218.

Sørensen, M. T., Vestergaard, E. M., Jensen, S. K., Lauridsen, C. and Højsgaard, S. (2009) 'Performance and diarrhoea in piglets following weaning at seven weeks of age: Challenge with E. coli O 149 and effect of dietary factors', *Livestock Science,* 123(2), pp. 314-321. DOI: 10.1016/j.livsci.2008.12.001.

Sorlozano-Puerto, A., Albertuz-Crespo, M., Lopez-Machado, I., Ariza-Romero, J. J., Baños-Arjona, A., Exposito-Ruiz, M. and Gutierrez-Fernandez, J. (2018) 'In vitro antibacterial activity of propyl-propane-thiosulfinate and propyl-propane-thiosulfonate derived from Allium spp. against gram-negative and gram-positive multidrug-resistant bacteria isolated from human samples', *BioMed Research International,* 2018(1), pp. 7861207.

Souillard, R., Répérant, J.-M., Experton, C., Huneau-Salaun, A., Coton, J., Balaine, L. and Le Bouquin, S. (2019) 'Husbandry Practices, Health, and Welfare Status of Organic Broilers in France', *Animals,* 9(3), pp. 97. Available at: https://www.mdpi.com/2076-2615/9/3/97.

Staba, E. J., Staba, J. E. and Lash, L. (2001) 'A Commentary on the Effects of Garlic Extraction and Formulation on Product Composition', *The Journal of Nutrition,* 131(3), pp. 1118S-1119S. DOI: 10.1093/jn/131.3.1118S.

Stachulski, A. V., Knausenberger, T. B. A., Shah, S. N., Hoyles, L. and McArthur, S. (2023) 'A host–gut microbial amino acid co-metabolite, p-cresol glucuronide, promotes blood–brain barrier integrity in vivo', *Tissue Barriers,* 11(1), pp. 2073175. DOI: 10.1080/21688370.2022.2073175.

Stefanello, C., Rosa, D. P., Dalmoro, Y. K., Segatto, A. L., Vieira, M. S., Moraes, M. L. and Santin, E. (2020) 'Protected Blend of Organic Acids and Essential Oils Improves Growth Performance, Nutrient Digestibility, and

Intestinal Health of Broiler Chickens Undergoing an Intestinal Challenge', *Frontiers in Veterinary Science,* 6. DOI: 10.3389/fvets.2019.00491.

Stefanello, C., Rosa, D.P., Santos, T.C., Rosa, A.P., Schwertz, C.I., Silva, W.T. and Radavelli, W.M., 2020. Protected blend of organic acids and essential oils in broilers challenged with Escherichia coli. *Frontiers in Veterinary Science*, 6, p.491. DOI:10.3389/fvets.2019.00491.

Sunanta, P., Kontogiorgos, V., Pankasemsuk, T., Jantanasakulwong, K., Rachtanapun, P., Seesuriyachan, P. and Sommano, S. R. (2023) 'The nutritional value, bioactive availability and functional properties of garlic and its related products during processing', *Frontiers in Nutrition,* 10. DOI: 10.3389/fnut.2023.1142784.

Tabart, J., Kevers, C., Pincemail, J., Defraigne, J.-O. and Dommes, J. (2006) 'Antioxidant Capacity of Black Currant Varies with Organ, Season, and Cultivar', *Journal of Agricultural and Food Chemistry,* 54(17), pp. 6271-6276. DOI: 10.1021/jf061112y.

Takahashi, S., Yoshida, Y., Nakanishi, N., Tsukahara, T. and Ushida, K. (2008) 'Quantitative real-time PCR monitoring of Escherichia coli and Clostridium perfringens with oral administration of Lactobacillus plantarum strain Lq80 to weaning piglets', *Animal Science Journal,* 79(6), pp. 737-744. DOI: 10.1111/j.1740-0929.2008.00588.x.

Tan, D., Zhang, Y., Chen, L., Liu, L., Zhang, X., Wu, Z., Bai, B. and Ji, S. (2015) 'Decreased glycation and structural protection properties of γ-glutamyl-S-allyl-cysteine peptide isolated from fresh garlic scales (Allium sativum L.)', *Natural Product Research,* 29(23), pp. 2219-2222.

Tan, J. B. L. and Lim, Y. Y. (2015) 'Critical analysis of current methods for assessing the in vitro antioxidant and antibacterial activity of plant extracts', *Food Chemistry,* 172, pp. 814-822. DOI: 10.1016/j.foodchem.2014.09.141.

Tebani, A. and Bekri, S. (2019) 'Paving the Way to Precision Nutrition Through Metabolomics', *Front Nutr,* 6, pp. 41. DOI: 10.3389/fnut.2019.00041.

Theilmann, M. C., Goh, Y. J., Nielsen, K. F., Klaenhammer, T. R., Barrangou, R. and Abou Hachem, M. (2017) 'Lactobacillus acidophilus Metabolizes Dietary Plant Glucosides and Externalizes Their Bioactive Phytochemicals', *mBio,* 8(6). DOI: 10.1128/mBio.01421-17.

Thormar, H. (2010) 'Lipids and essential oils as antimicrobial agents'.

Thumann, T. A., Pferschy-Wenzig, E.-M., Moissl-Eichinger, C. and Bauer, R. (2019) 'The role of gut microbiota for the activity of medicinal plants traditionally used in the European Union for gastrointestinal disorders', *Journal of Ethnopharmacology,* 245, pp. 112153. DOI: 10.1016/j.jep.2019.112153.

Thuy, N. T. and Ha, N. C. (2023) 'Effect of adding guava leaf (Psidium guajava) and garlic (Allium sativum) powders in diets on growth performance and diarrhea incidence of weaned piglets', *Livest Res Rural Dev,* 35.

Tian, Y., Laaksonen, O., Haikonen, H., Vanag, A., Ejaz, H., Linderborg, K., Karhu, S. and Yang, B. (2019) 'Compositional Diversity among Blackcurrant (Ribes nigrum) Cultivars Originating from European Countries', *Journal of Agricultural and Food Chemistry,* 67(19), pp. 5621-5633. DOI: 10.1021/acs.jafc.9b00033.

Timbermont, L., Lanckriet, A., Dewulf, J., Nollet, N., Schwarzer, K., Haesebrouck, F., Ducatelle, R. and Van Immerseel, F. (2010) 'Control of Clostridium perfringens-induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils', *Avian Pathology,* 39(2), pp. 117-121.

Tipton, L., Darcy, J. L. and Hynson, N. A. (2019) 'A Developing Symbiosis: Enabling Cross-Talk Between Ecologists and Microbiome Scientists', *Frontiers in Microbiology,* 10. DOI: 10.3389/fmicb.2019.00292.

To, H., Suzuki, T., Kawahara, F., Uetsuka, K., Nagai, S. and Nunoya, T. (2017) 'Experimental induction of necrotic enteritis in chickens by a *netB*</i>-positive Japanese isolate of Clostridium perfringens</i>', *Journal of Veterinary Medical Science,* 79(2), pp. 350-358. DOI: 10.1292/jvms.16-0500.

Tudela, H., Claus, S. P. and Saleh, M. (2021) 'Next Generation Microbiome Research: Identification of Keystone Species in the Metabolic Regulation of Host-Gut Microbiota Interplay', *Frontiers in Cell and Developmental Biology,* 9. DOI: 10.3389/fcell.2021.719072.

Tudu, C. K., Dutta, T., Ghorai, M., Biswas, P., Samanta, D., Oleksak, P., Jha, N. K., Kumar, M., Radha, Proćków, J., Pérez de la Lastra, J. M. and Dey, A. (2022) 'Traditional uses, phytochemistry, pharmacology and toxicology of garlic (Allium sativum), a storehouse of diverse phytochemicals: A review of research from the last decade focusing on health and nutritional implications', *Frontiers in Nutrition,* 9. DOI: 10.3389/fnut.2022.929554.

Tugnoli, B., Giovagnoni, G., Piva, A. and Grilli, E. (2020) 'From acidifiers to intestinal health enhancers: How organic acids can improve growth efficiency of pigs', *Animals,* 10(1), pp. 134.

Valdes, A. M., Walter, J., Segal, E. and Spector, T. D. (2018) 'Role of the gut microbiota in nutrition and health', *BMJ,* 361, pp. k2179. DOI: 10.1136/bmj.k2179.

Valdez, G., Shyur, L.-F., Wang, S.-Y. and Chen, S.-E. (2023) 'Phytogenics in Ginger, Origanum vulgare, and Syzygium aromaticum and Their Potential as a Feed Additive against Clostridium perfringens in Broiler Production', *Animals,* 13(23), pp. 3643. Available at: https://www.mdpi.com/2076-2615/13/23/3643.

van Eerden, E., Santos, R. R., Molist, F., Dardi, M., Pantoja-Millas, L. A., Molist-Badiola, J., Baratelli, M. and Pages, M. (2022) 'Efficacy of an attenuated vaccine against avian coccidiosis in combination with feed additives based on organic acids and essential oils on production performance and intestinal lesions in broilers experimentally challenged with necrotic enteritis', *Poultry Science,* 101(6), pp. 101848. DOI: 10.1016/j.psj.2022.101848.

Van Immerseel, F., Rood, J. I., Moore, R. J. and Titball, R. W. (2009) 'Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens', *Trends in Microbiology,* 17(1), pp. 32-36. DOI: 10.1016/j.tim.2008.09.005.

van Vuuren, S. and Viljoen, A. (2011) 'Plant-Based Antimicrobial Studies – Methods and Approaches to Study the Interaction between Natural Products', *Planta Med,* 77(11), pp. 1168-1182. DOI: 10.1055/s-0030-1250736.

Vaou, N., Stavropoulou, E., Voidarou, C. C., Tsakris, Z., Rozos, G., Tsigalou, C. and Bezirtzoglou, E. (2022) 'Interactions between Medical Plant-Derived Bioactive Compounds: Focus on Antimicrobial Combination Effects', *Antibiotics (Basel),* 11(8). DOI: 10.3390/antibiotics11081014.

Vasquez, R., Oh, J. K., Song, J. H. and Kang, D. K. (2022) 'Gut microbiome-produced metabolites in pigs: a review on their biological functions and the influence of probiotics', *J Anim Sci Technol,* 64(4), pp. 671-695. DOI: 10.5187/jast.2022.e58.

Vermaak, I., Viljoen, A. M., Hamman, J. H. and Van Vuuren, S. F. (2009) 'The effect of simulated gastrointestinal conditions on the antimicrobial activity and chemical composition of indigenous South African plant extracts', *South African Journal of Botany,* 75(3), pp. 594-599. DOI: 10.1016/j.sajb.2009.02.180.

Vermeire, B., Gonzalez, L. M., Jansens, R. J. J., Cox, E. and Devriendt, B. (2021) 'Porcine small intestinal organoids as a model to explore ETEC-host interactions in the gut', *Vet Res,* 52(1), pp. 94. DOI: 10.1186/s13567-021-00961-7.

Wagner, H. and Ulrich-Merzenich, G. (2009) 'Synergy research: Approaching a new generation of phytopharmaceuticals', *Phytomedicine,* 16(2), pp. 97-110. DOI: 10.1016/j.phymed.2008.12.018.

Waldbauer, K., McKinnon, R. and Kopp, B. (2017) 'Apple Pomace as Potential Source of Natural Active Compounds', *Planta Med,* 83(12/13), pp. 994-1010. DOI: 10.1055/s-0043-111898.

Walton, M. C., Lentle, R. G., Reynolds, G. W., Kruger, M. C. and McGhie, T. K. (2006) 'Anthocyanin Absorption and Antioxidant Status in Pigs', *Journal of Agricultural and Food Chemistry,* 54(20), pp. 7940-7946. DOI: 10.1021/jf061527j.

Wang GuoJun, W. G., Gao Yin, G. Y., Lai HangXian, L. H. and Yang YuXin, Y. Y. (2017) 'Effects of probiotic fermented apple pomace on the apparent digestibility, serum immune indexes, intestinal microflora and morphology in weaned piglets'.

Wang, J. P., Yoo, J. S., Jang, H. D., Lee, J. H., Cho, J. H. and Kim, I. H. (2011) 'Effect of dietary fermented garlic by Weissella koreensis powder on growth performance, blood characteristics, and immune response of growing pigs challenged with Escherichia coli lipopolysaccharide', *Journal of Animal Science,* 89(7), pp. 2123-2131. DOI: 10.2527/jas.2010-3186.

Wang, L. F., Bergstrom, J. R., Hahn, J. D., Young, M. G. and Zijlstra, R. T. (2023) 'Acid-binding capacity of feed in swine nutrition', *Animal Feed Science and Technology,* 295, pp. 115519. DOI: 10.1016/j.anifeedsci.2022.115519.

Wang, W. and Gänzle, M. (2019) 'Chapter Three - Toward rational selection criteria for selection of probiotics in pigs', in Gadd, G.M. & Sariaslani, S. (eds.) *Advances in Applied Microbiology*. Academic Press, pp. 83-112.

Wang, W., Zijlstra, R. T. and Gänzle, M. G. (2017) 'Identification and quantification of virulence factors of enterotoxigenic Escherichia coli by high-resolution melting curve quantitative PCR', *BMC Microbiology,* 17(1), pp. 114. DOI: 10.1186/s12866-017-1023-5.

Weber, N. R., Nielsen, J. P., Hjulsager, C. K., Jorsal, S. E., Haugegaard, S., Hansen, C. F. and Pedersen, K. S. (2017) 'Comparison of bacterial culture and qPCR testing of rectal and pen floor samples as diagnostic approaches to detect enterotoxic Escherichia coli in nursery pigs', *Preventive Veterinary Medicine,* 143, pp. 61-67. DOI: 10.1016/j.prevetmed.2017.05.009.

Wenk, C. (2001) 'The role of dietary fibre in the digestive physiology of the pig', *Animal Feed Science and Technology,* 90(1), pp. 21-33. DOI: 10.1016/S0377-8401(01)00194-8.

Wenk, C. (2003) 'Herbs and botanicals as feed additives in monogastric animals', *Asian-Australasian Journal of Animal Sciences,* 16(2), pp. 282-289.

Wierup, M. (2001) 'The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials', *Microbial drug resistance,* 7(2), pp. 183-190.

Williams, R. B. (2005) 'Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity', *Avian Pathology,* 34(3), pp. 159-180. DOI: 10.1080/03079450500112195.

Windisch, W., Schedle, K., Plitzner, C. and Kroismayr, A. (2008) 'Use of phytogenic products as feed additives for swine and poultry1', *Journal of Animal Science,* 86(suppl_14), pp. E140-E148. DOI: 10.2527/jas.2007-0459.

Wlaźlak, S., Pietrzak, E., Biesek, J. and Dunislawska, A. (2023) 'Modulation of the immune system of chickens a key factor in maintaining poultry production—a review', *Poultry Science,* 102(8), pp. 102785. DOI: 10.1016/j.psj.2023.102785.

Wong, B. T., Park, S., Kovanda, L., He, Y., Kim, K., Xu, S., Lingga, C., Hejna, M., Wall, E., Sripathy, R., Li, X. and Liu, Y. (2022) 'Dietary supplementation of botanical blends enhanced performance and disease resistance of weaned pigs experimentally infected with enterotoxigenic Escherichia coli F18', *J Anim Sci,* 100(12). DOI: 10.1093/jas/skac353.

Wu, C., Wang, M., Cheng, Z. and Meng, H. (2016) 'Response of garlic (Allium sativum L.) bolting and bulbing to temperature and photoperiod treatments', *Biol Open,* 5(4), pp. 507-18. DOI: 10.1242/bio.016444.

Wu, X., Pittman, H. E., McKay, S. and Prior, R. L. (2005) 'Aglycones and Sugar Moieties Alter Anthocyanin Absorption and Metabolism after Berry Consumption in Weanling Pigs12', *The Journal of Nutrition,* 135(10), pp. 2417-2424. DOI: 10.1093/jn/135.10.2417.

Xu, Y., Lahaye, L., He, Z., Zhang, J., Yang, C. and Piao, X. (2020) 'Micro-encapsulated essential oils and organic acids combination improves intestinal barrier function, inflammatory responses and microbiota of weaned piglets challenged with enterotoxigenic Escherichia coli F4 (K88+)', *Animal nutrition,* 6(3), pp. 269-277.

Xue, B., Hui, X., Chen, X., Luo, S., Dilrukshi, H. N. N., Wu, G. and Chen, C. (2022) 'Application, emerging health benefits, and dosage effects of blackcurrant food formats', *Journal of Functional Foods,* 95, pp. 105147. DOI: 10.1016/j.jff.2022.105147.

Yan, L. and Kim, I. H. (2013) 'Effects of dietary supplementation of fermented garlic powder on growth performance, apparent total tract digestibility, blood characteristics and faecal microbial concentration in weanling pigs', *Journal of Animal Physiology and Animal Nutrition,* 97(3), pp. 457-464. DOI: 10.1111/j.1439-0396.2012.01286.x.

Yang, H.-T., Chen, J.-W., Rathod, J., Jiang, Y.-Z., Tsai, P.-J., Hung, Y.-P., Ko, W.-C., Paredes-Sabja, D. and Huang, I.-H. (2018) 'Lauric Acid Is an Inhibitor of Clostridium difficile Growth in Vitro and Reduces Inflammation in a Mouse Infection Model', *Frontiers in Microbiology,* 8. DOI: 10.3389/fmicb.2017.02635.

Yang, W.-Y., Lee, Y., Lu, H., Chou, C.-H. and Wang, C. (2019b) 'Analysis of gut microbiota and the effect of lauric acid against necrotic enteritis in Clostridium perfringens and Eimeria side-by-side challenge model', *PLOS ONE,* 14(5), pp. e0205784. DOI: 10.1371/journal.pone.0205784.

Yang, W.-Y., Lee, Y.-J., Lu, H.-Y., Branton, S. L., Chou, C.-H. and Wang, C. (2019a) 'The *netB*-positive Clostridium perfringens in the experimental induction of necrotic enteritis with or without predisposing factors', *Poultry Science,* 98(11), pp. 5297-5306. DOI: 10.3382/ps/pez311.

Yang, Y., Wang, Q., Diarra, M. S., Yu, H., Hua, Y. and Gong, J. (2016) 'Functional assessment of encapsulated citral for controlling necrotic enteritis in broiler chickens', *Poultry Science,* 95(4), pp. 780-789. DOI: 10.3382/ps/pev375.

Yaqoob, M. U., Wang, G. and Wang, M. (2022) 'An updated review on probiotics as an alternative of antibiotics in poultry - A review', *Anim Biosci,* 35(8), pp. 1109-1120. DOI: 10.5713/ab.21.0485.

Yin, M.-c. and Cheng, W.-s. (2003) 'Antioxidant and antimicrobial effects of four garlic-derived organosulfur compounds in ground beef', *Meat Science,* 63(1), pp. 23-28. DOI: 10.1016/S0309-1740(02)00047-5.

Yoon, B. K., Jackman, J. A., Kim, M. C. and Cho, N.-J. (2015) 'Spectrum of Membrane Morphological Responses to Antibacterial Fatty Acids and Related Surfactants', *Langmuir,* 31(37), pp. 10223-10232. DOI: 10.1021/acs.langmuir.5b02088.

Yoon, B. K., Jackman, J. A., Valle-González, E. R. and Cho, N. J. (2018) 'Antibacterial Free Fatty Acids and Monoglycerides: Biological Activities, Experimental Testing, and Therapeutic Applications', *Int J Mol Sci,* 19(4). DOI: 10.3390/ijms19041114.

Young, F. (1983) 'Palm kernel and coconut oils: analytical characteristics, process technology and uses', *Journal of the American Oil Chemists' Society,* 60(2Part2), pp. 374-379.

Yu, Q., Lepp, D., Mehdizadeh Gohari, I., Wu, T., Zhou, H., Yin, X., Yu, H., Prescott, J. F., Nie, S. P., Xie, M. Y. and Gong, J. (2017) 'The Agr-Like Quorum Sensing System Is Required for Pathogenesis of Necrotic Enteritis Caused by Clostridium perfringens in Poultry', *Infect Immun,* 85(6). DOI: 10.1128/iai.00975-16.

Zambrano, C., Kerekes, E. B., Kotogán, A., Papp, T., Vágvölgyi, C., Krisch, J. and Takó, M. (2019) 'Antimicrobial activity of grape, apple and pitahaya residue extracts after carbohydrase treatment against food-related bacteria', *LWT,* 100, pp. 416-425. DOI: 10.1016/j.lwt.2018.10.044.

Zampiga, M., Flees, J., Meluzzi, A., Dridi, S. and Sirri, F. (2018) 'Application of omics technologies for a deeper insight into quali-quantitative production traits in broiler chickens: A review', *Journal of Animal Science and Biotechnology,* 9. DOI: 10.1186/s40104-018-0278-5.

Zeitz, J. O., Fennhoff, J., Kluge, H., Stangl, G. I. and Eder, K. (2015) 'Effects of dietary fats rich in lauric and myristic acid on performance, intestinal morphology, gut microbes, and meat quality in broilers', *Poultry Science,* 94(10), pp. 2404-2413. DOI: 10.3382/ps/pev191.

Zentek, J., Buchheit-Renko, S., Ferrara, F., Vahjen, W., Van Kessel, A. G. and Pieper, R. (2011) 'Nutritional and physiological role of medium-chain triglycerides and medium-chain fatty acids in piglets', *Animal Health Research Reviews,* 12(1), pp. 83-93. DOI: 10.1017/S1466252311000089.

Zentek, J., Ferrara, F., Pieper, R., Tedin, L., Meyer, W. and Vahjen, W. (2013) 'Effects of dietary combinations of organic acids and medium chain fatty acids on the gastrointestinal microbial ecology and bacterial metabolites in the digestive tract of weaning piglets1', *Journal of Animal Science,* 91(7), pp. 3200-3210. DOI: 10.2527/jas.2012-5673.

Zhai, H., Liu, H., Wang, S., Wu, J. and Kluenter, A.-M. (2018) 'Potential of essential oils for poultry and pigs', *Animal Nutrition,* 4(2), pp. 179-186. DOI: 10.1016/j.aninu.2018.01.005.

Zhang, F., Wang, T., Wang, X. and Lü, X. (2021) 'Apple pomace as a potential valuable resource for full-components utilization: A review', *Journal of Cleaner Production,* 329, pp. 129676. DOI: 10.1016/j.jclepro.2021.129676.

Zhang, T., Wei, X., Miao, Z., Hassan, H., Song, Y. and Fan, M. (2016) 'Screening for antioxidant and antibacterial activities of phenolics from Golden Delicious apple pomace', *Chemistry Central Journal,* 10(1), pp. 47. DOI: 10.1186/s13065-016-0195-7.

Zhang, Y., Gong, J., Yu, H., Guo, Q., Defelice, C., Hernandez, M., Yin, Y. and Wang, Q. (2014) 'Alginate-whey protein dry powder optimized for target delivery of essential oils to the intestine of chickens', *Poultry Science,* 93(10), pp. 2514-2525. DOI: 10.3382/ps.2013-03843.

Zhang, Y., Liu, X., Ruan, J., Zhuang, X., Zhang, X. and Li, Z. (2020) 'Phytochemicals of garlic: Promising candidates for cancer therapy', *Biomedicine & Pharmacotherapy,* 123, pp. 109730. DOI: 10.1016/j.biopha.2019.109730.

Zhao, R., Qiu, Z., Bai, X., Xiang, L., Qiao, Y. and Lu, X. (2022) 'Digestive properties and prebiotic activity of garlic saccharides with different-molecular-weight obtained by acidolysis', *Current Research in Food Science,* 5, pp. 2033-2044.

Zheng, J., Yang, B., Ruusunen, V., Laaksonen, O., Tahvonen, R., Hellsten, J. and Kallio, H. (2012) 'Compositional Differences of Phenolic Compounds between Black Currant (Ribes nigrum L.) Cultivars and Their Response to Latitude and Weather Conditions', *Journal of Agricultural and Food Chemistry,* 60(26), pp. 6581-6593. DOI: 10.1021/jf3012739.

Zi, C., Zeng, D., Ling, N., Dai, J., Xue, F., Jiang, Y. and Li, B. (2018) 'An improved assay for rapid detection of viable Staphylococcus aureus cells by incorporating surfactant and PMA treatments in qPCR', *BMC Microbiology,* 18(1), pp. 132. DOI: 10.1186/s12866-018-1273-x.