Norwegian University of Life Sciences
Faculty of Veterinary Medicine

Philosophiae Doctor (PhD)
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Impact of feed composition on rearing of broiler chickens without in-feed antimicrobials

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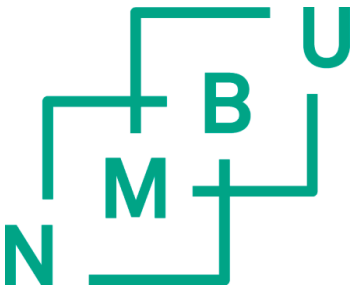

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Abbreviations and acronyms

AGPs	Antibiotic growth promoters
AMR	Antimicrobial resistance
ATAs	Alternatives to antibiotics
CDC	Centers for Disease Control and Prevention
cpa	*Clostridium perfringens* alpha toxin gene
ddCFU	Droplet digital (colony-forming-unit) assays
ddPCR	Droplet digital polymerase chain reaction
dPCR	Digital polymerase chain reaction
EU	European Union
EIP-AGRI	European Innovation Partnership for Agricultural Productivity and Sustainability
FAO	Food and Agriculture Organization of the United Nations
FDA	U.S. Food and Drug Administration
FVE	Federation of Veterinarians of Europe
HS	High starch to fat ratio
LMD	The Norwegian Ministry of Agriculture and Food
LS	Low starch to fat ratio
netB	*Clostridium perfringens* necrotic enteritis B-like toxin gene
NORM-VET	Norwegian Veterinary Antimicrobial Resistance Monitoring
NSPs	Non-starch polysaccharides
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
qPCR	Quantitative real-time polymerase chain reaction
SCFAs	Short-chain fatty acids
UK	United Kingdom
UN	United Nations
US	United States of America
VKM	Norwegian Scientific Committee for Food and Environment
WHO	World Health Organization

List of papers

Paper I

Effect of feed additives as alternatives to in-feed antimicrobials on production performance and intestinal *Clostridium perfringens* counts in broiler chickens

Granstad S, Kristoffersen AB, Benestad SL, Sjurseth SK, David B, Sørensen L, Fjermedal A, Edvardsen DH, Sanson G, Løvland A and Kaldhusdal M.

Animals 2020, 10, 240; doi:10.3390/ani10020240

Paper II

Varying starch to fat ratios in pelleted diets: I. Effects on nutrient digestibility and production performance in *Eimeria*-challenged broiler chickens

Itani K*, Granstad S*, Kaldhusdal M, Mydland LT and Svihus B.

* Shared first authorship

British Poultry Science 2020, 61:6, 703-709; doi:10.1080/00071668.2020.1782349

Paper III

Varying starch to fat ratios in pelleted diets: II. Effects on intestinal histomorphometry, *Clostridium perfringens* and short-chain fatty acids in *Eimeria*-challenged broiler chickens

Granstad S, Itani K, Benestad SL, Øines Ø, Svihus B and Kaldhusdal M.

British Poultry Science 2021, 62:1, 92-100; doi:10.1080/00071668.2020.1810630

Summary

In-feed antimicrobials with antibiotic properties have been used for disease-preventive and performance-promoting purposes in rearing of broiler chickens since the mid-twentieth century. The use of antibiotic agents in subtherapeutic doses to promote growth has later been banned in several countries. However, ionophorous polyether antibiotics ('ionophores') with anticoccidial and antibacterial properties are still extensively used in broiler production in most countries, except Norway, to control the key production-limiting diseases coccidiosis and *Clostridium perfringens*-associated necrotic enteritis.

Antimicrobial resistance is a serious threat to global public health, and the increasingly higher occurrence of antibiotic resistant pathogens requires urgent action and prudent use of antibiotics. Increased consumer awareness and criticism from other stakeholders, novel therapeutic indications of ionophores and a possible association between ionophores and antimicrobial resistance to antibiotics important in human medicine, may lead to future restrictions of the prophylactic use of ionophores in more countries.

The aim of this thesis was to study and assess specific dietary manipulations in broiler chickens reared in 'antimicrobial-free' production systems, meaning that no substances with antibiotic properties were added to the feed. Effects of adding non-antibiotic feed additives or changing the dietary ratio of starch to fat on intestinal health and production performance in Ross 308 broilers were investigated by the use of an experimental animal model simulating commercial broiler house conditions.

The first hypothesis of this work was that diets supplied with non-antibiotic feed additive products improve production performance and/or reduce intestinal *C. perfringens* counts compared with a diet with no feed additives. Twenty commercially available feed additives categorized as prebiotics, probiotics, phytogenics and/or organic acid-based products, and four combinations of such products, were evaluated based on their effect on caecal *C. perfringens* counts and production performance. Probiotic feed additives with *Bacillus subtilis* strains improved production results in a time interval of the study comprising infection with coccidia, and one of these products

concurrently reduced *C. perfringens* counts. Prebiotic feed additives, all based on non-living components from the yeast *Saccharomyces cerevisiae*, improved production performance and particularly feed conversion ratio in the rearing interval with coccidia infection. The heterogeneous group of feed additives with phytogenic compounds showed variable results. A product based on oleoresins from turmeric and chilli peppers improved overall feed conversion and reduced *C. perfringens* counts, and two products containing essential oils improved production performance in the time interval comprising infection with coccidia. Several organic acid-based feed additives containing short- and/or medium-chain fatty acids or derivatives thereof improved overall production performance, and demonstrated superior production performance results during the first two weeks post-hatch. In general, the study of non-antibiotic feed additives provided comparable and unbiased results from testing of a representative selection of commercially available products and product combinations in a uniform experimental model relevant to commercial rearing of broilers. The different non-antibiotic feed additives affected production performance and intestinal *C. perfringens* counts with varying degrees of success, and some feed additives had beneficial impact only during distinct rearing phases and on specific performance targets. The use of optimized combinations of different active components with synergistic effects, and targeted use of feed additives in specific phases of the production cycle, are promising strategies in antimicrobial-free broiler rearing systems.

The second hypothesis was that a high dietary starch to fat ratio impairs starch digestibility, production performance and intestinal health in broilers challenged with coccidia. Two antimicrobial-free diets with similar energy and protein content, but with different inclusion level of starch and fat, were evaluated for their effect on nutrient digestibility, production performance, small intestinal morphology, *C. perfringens* counts and toxin profile, necrotic enteritis prevalence and caecal short-chain fatty acid (SCFA) abundances in coccidia-challenged broilers. Results showed that the diet with the highest starch inclusion level augmented starch digestibility, led to improved feed conversion and did not have an impact on intestinal *C. perfringens* counts, toxin profile or necrotic enteritis prevalence. Thus, the hypothesis that a higher dietary starch to fat ratio would impair intestinal health and production performance was not verified. A rejection of the hypothesis needs to take two possibly confounding factors of the high

starch to fat ratio diet into consideration: The degree of starch gelatinization and the use of isolated wheat starch. In chickens fed the high starch to fat ratio diet, total caecal SCFA level was greater prior to infection with coccidia and small intestinal villi were longer after infection with coccidia. The study of dietary starch to fat ratios suggests that broiler chickens have the capacity to adapt to different diet compositions by structural remodelling of the small intestine, and that chickens adapted to higher levels of dietary starch might be more resilient to *Eimeria* spp. infections as a consequence of increased mucosal surface area.

Possible future restrictions of the use of ionophores in the global broiler industry call for more knowledge of the impact of feed composition on development of intestinal disease and production performance. Results from this thesis suggest that some non-antibiotic feed additives have disease-preventive and performance-enhancing effects. Further, diets with a high starch to fat ratio do not seem to predispose for intestinal disease if the dietary starch is readily available and easily digested.

Sammendrag (Summary in Norwegian)

Antimikrobielle midler med antibiotiske egenskaper har blitt tilsatt i fôret til slaktekylling siden midten av det 20. århundre for å forebygge sykdom og fremme ytelse. Bruk av antibiotika i subterapeutiske doser for å fremme tilvekst har senere blitt forbudt i flere land, men ionofor-polyeterantibiotika ('ionoforer') med effekt mot koksidier og bakterier benyttes fortsatt i stort omfang i de fleste land med unntak av Norge. Ionoforer brukes hovedsakelig for å forhindre koksidiose og *Clostridium perfringens*-assosiert nekrotiserende enteritt, som er blant de mest tapsbringende sykdommene hos slaktekylling.

Antibiotikaresistens er en alvorlig trussel mot global folkehelse, og stadig økende forekomst av antibiotikaresistente sykdomsfremkallende bakterier krever umiddelbar handling og restriktiv bruk av antibiotika. Økt forbrukerbevissthet og kritikk fra ulike interessegrupper, nye terapeutiske indikasjoner for ionoforer og en mulig sammenheng mellom ionoforer og resistens mot antibiotika som er viktige i humanmedisinen, vil kunne føre til fremtidige restriksjoner på profylaktisk bruk av ionoforer i flere land.

Formålet med doktorgradsarbeidet var å studere og evaluere spesifikke endringer i fôrsammensetningen i slaktekyllingoppdrett uten bruk av antimikrobielle midler med antibiotiske egenskaper i fôret. Effekten av ikke-antibiotiske fôrtilsetninger og betydningen av å endre mengdeforholdet mellom stivelse og fett i fôret på tarmhelse og produksjonsresultat hos Ross 308 slaktekyllinger, ble undersøkt ved hjelp av en dyreforsøksmodell som simulerte forholdene i kommersielle kyllinghus.

Den første hypotesen i arbeidet var at fôr supplert med ikke-antibiotiske fôrtilsetninger forbedrer produksjonsresultatet og/eller reduserer antall *C. perfringens* i tarmen sammenlignet med et fôr uten fôrtilsetninger. Tjue kommersielt tilgjengelige fôrtilsetninger kategorisert som prebiotika, probiotika, planteprodukter og/eller produkter basert på organiske syrer, og fire kombinasjoner av slike produkter, ble evaluert basert på deres innvirkning på antall *C. perfringens* i blindtarm og produksjonsresultater. Probiotiske fôrtilsetninger med *Bacillus subtilis*-stammer forbedret produksjonsresultatene i et tidsintervall av studien som inkluderte infeksjon

med koksidier, og ett av disse produktene reduserte også antall *C. perfringens* i samme tidsintervall. Prebiotiske fôrtilsetninger, som alle var basert på ikke-levende komponenter fra gjærsopparten *Saccharomyces cerevisiae*, forbedret produksjonsresultater og spesielt fôrutnyttelsen i fasen av oppdrettet med koksidieinfeksjon. Den heterogene gruppen av plantebaserte fôrtilsetninger viste varierende resultater. Et produkt basert på oleoresiner fra gurkemeie og chili forbedret fôrutnyttelsen for hele forsøksperioden og reduserte antall *C. perfringens*. To produkter som inneholdt eteriske oljer forbedret produksjonsresultater i perioden som inkluderte infeksjon med koksidier. Flere fôrtilsetninger basert på organiske syrer som inneholdt korte og/eller mellomlange fettsyrer eller derivater av disse, forbedret produksjonsresultatene for hele studieperioden. Slike produkter genererte også svært gode produksjonsresultater i de to første ukene etter klekking. Studien av ikke-antibiotiske fôrtilsetninger genererte sammenlignbare og objektive resultater fra testing av et representativt utvalg av kommersielt tilgjengelige produkter og produktkombinasjoner i en standardisert forsøksmodell relevant for kommersielt slaktekyllingoppdrett. De ulike ikke-antibiotiske fôrtilsetningene påvirket produksjonsresultat og antall *C. perfringens* i blindtarm med varierende grad av suksess, og enkelte fôrtilsetninger hadde gunstig effekt kun under bestemte faser av oppdrettet eller på spesifikke ytelsesparametere. Bruk av optimaliserte kombinasjoner av ulike aktive komponenter med synergistiske effekter, samt målrettet bruk av fôrtilsetninger i spesifikke faser av produksjonssyklusen, er potensielle strategier i slaktekyllingoppdrett uten bruk av antimikrobielle midler.

Den andre hypotesen var at en høy stivelse/fett-ratio i fôret svekker stivelsesfordøyeligheten, produksjonsresultatene og tarmhelsen hos slaktekylling som utsettes for infeksjon med koksidier. To antibiotika-frie dietter med likt energi- og proteininnhold, men med ulikt innhold av stivelse og fett, ble evaluert på grunnlag av deres effekt på fordøyelighet av næringsstoffer, produksjonsresultat, tynntarmsmorfologi, *C. perfringens*-tall og toksinprofil, forekomst av nekrotiserende enteritt og mengde kortkjedede fettsyrer i blindtarm hos koksidiesmittede slaktekyllinger. Resultatene viste at fôret med høyest innhold av stivelse økte stivelsesfordøyeligheten, førte til forbedret fôrutnyttelse og ikke hadde noen innvirkning på antall *C. perfringens*, toksinprofil eller forekomst av nekrotiserende

enteritt. Dermed ble ikke hypotesen om at en høy stivelse/fett-ratio svekker tarmhelse og produksjonsresultat verifisert. Dersom hypotesen skal forkastes må to potensielt konfunderende faktorer i fôret med høy stivelse/fett-ratio tas i betraktning: Grad av forklistring og bruk av isolert hvetestivelse. I kyllinger som fikk fôret med høy stivelse/fett-ratio var den totale mengden kortkjedede fettsyrer i blindtarm høyere før koksidieinfeksjon, og lengden på villi i tynntarm var lengre etter koksidieinfeksjon. Studien av fôr med ulik stivelse/fett-ratio indikerer at slaktekyllinger har kapasitet til å tilpasse seg ulike dietter ved strukturell remodellering av tynntarmen. Videre antyder funnene i studien at kyllinger tilpasset en høyere mengde stivelse i fôret kan være mer robuste mot koksidieinfeksjoner grunnet økt overflateareal av tarmens slimhinne.

Mulige fremtidige restriksjoner relatert til den profylaktiske bruken av ionoforer i den globale slaktekyllingnæringen krever mer kunnskap om betydningen av fôrsammensetning for sykdomsutvikling og produksjonsresultat. Resultater fra dette doktorgradsarbeidet indikerer at enkelte ikke-antibiotiske fôrtilsetninger har sykdomsforebyggende og ytelsesfremmende effekt. Videre viser resultatene at dietter med høy stivelse/fett-ratio ikke ser ut til å predisponere for tarmsykdom dersom stivelsen i fôret er tilgjengelig og lettfordøyelig.

1. Introduction

1.1 Global challenges in livestock industries

The livestock sector has evolved substantially during the past decades. Progress in breeding, nutrition and animal health has contributed to increased efficiency and production volumes (Thornton, 2010). Extensive growth of the human population calls for further refinements of resource utilization in livestock farming, as hunger, undernutrition and food insecurity are still significant concerns in many parts of the world. Estimates done by the United Nations (UN) show that more than 690 million people were undernourished in 2019, and the COVID-19 pandemic is expected to put additional millions of people at risk of suffering from acute hunger (United Nations, 2021). The UN Sustainable Development Goal 2 aims to end hunger and malnutrition, but if current trends continue, the goal will not be achieved by 2030. This implies a global demand for more efficient use of natural resources and improvements in food and feed production.

Poultry meat production is one of the fastest growing sectors in agriculture worldwide, and the global production volume has increased more than 14-fold between the years 1961-2018 (Ritchie & Roser, 2017). In recent years, poultry meat has become the world's most significant meat type, exceeding the production volumes of the previously dominant pig meat industry (FAO, 2020). The world's largest producers of poultry meat (the United States, China and Brazil) are located on different continents, illustrating the global relevance of this meat type (Figure 1). The production and consumption volumes of poultry meat are expected to continue to grow in the future decades (Alexandratos & Bruinsma, 2012). Chickens have superior feed conversion rates compared to other food-producing terrestrial animals, and the broiler industry is characterized by relatively low production costs and rapid production cycles. Furthermore, production of chicken meat is considered to have the smallest environmental footprint measured in greenhouse gas emissions and use of natural resources compared with other terrestrial animal meat productions (de Vries & de Boer, 2010; Vaarst et al., 2015).

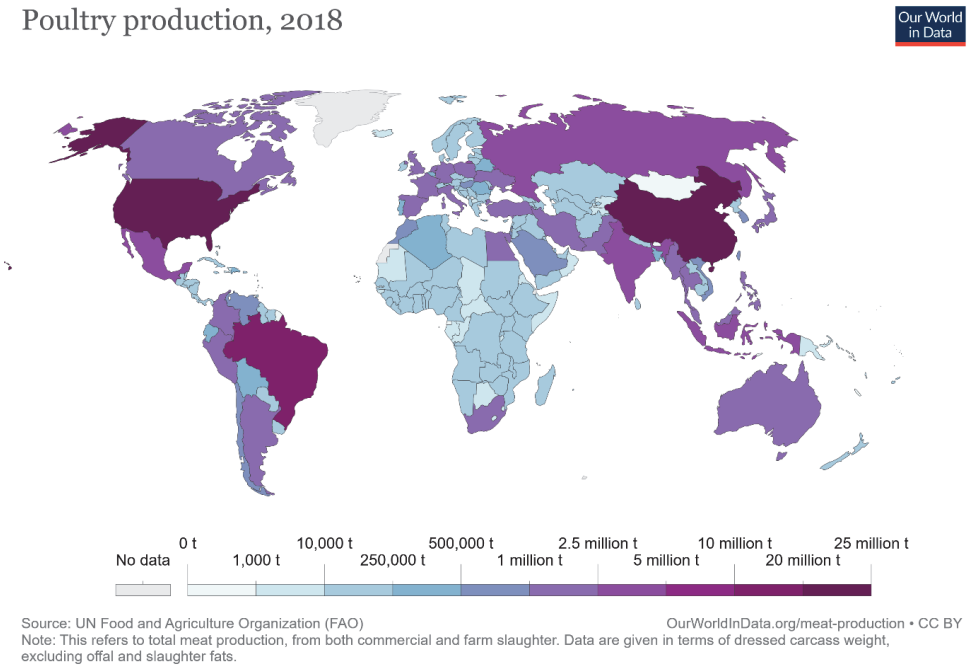

Figure 1. Global poultry meat production measured in tonnes in 2018. Based on data from the Food and Agriculture Organization of the United Nations (FAO). Retrieved from https://ourworldindata.org/meat-production (16.03.2021).

Maintenance of animal health and thus prevention of disease is fundamental to sustain welfare and profit in livestock farming. With an increasing demand for highly effective food production systems worldwide, in-feed antimicrobials have been and are still used both for treatment and prevention of disease in livestock industries (Manyi-Loh et al., 2018). In some countries, use of antimicrobials in livestock is primarily associated with the routine in-feed supplementation of antimicrobials as growth promoters or as a low-cost substitute for hygiene measures that could otherwise prevent infections. Inappropriate use of antimicrobials in animals is identified as one of the leading causes of rising antimicrobial resistance (Humphreys & Fleck, 2016; Van Boeckel et al., 2017). Antimicrobial resistance (AMR) is defined as the ability of microorganisms to proliferate in presence of an antimicrobial agent that generally inhibits or kills microorganisms of the same species, and is caused by genetic mutations or acquisition of resistance genes (Harbottle et al., 2006; WHO, 2020). As a consequence of AMR, infectious diseases in

humans and animals can be complicated, prolonged or impossible to treat, leading to higher medical costs and in worst case death.

Some of the most important global challenges in livestock industries are growing demands for nutritious food and increased productivity on one hand, and control of infectious diseases, use of antimicrobial agents and AMR on the other. The uncontrolled spread of antimicrobial resistant pathogens poses a significant global public health threat, and effective alternatives to control infectious agents are needed to safeguard important antibiotics and keep them as useful tools for future generations.

1.2 Antimicrobials in broiler production

Therapeutic use of antibiotics for treatment of diagnosed infections and prophylactic use of in-feed antimicrobials with antibiotic properties contribute to spread of AMR in broiler production systems worldwide. The latter use of antimicrobials has supported the extensive growth of the broiler chicken industry over the past decades. Categorization of antimicrobial agents is sometimes inconsistent. Hence, an overview of the terminology used in this thesis is presented in Table 1 (CDC, 2019; FVE, 2016; Noack et al., 2019; VKM, 2015; Wegener et al., 1999).

Table 1. Definitions of key terms related to antimicrobial agents.

Term	Definition
Antimicrobials	Substances of natural, semisynthetic or synthetic origin that kill or inhibit growth of microorganisms (bacteria, viruses, parasites and/or fungi).
Therapeutic antibiotics	Antimicrobial drugs used to treat bacterial infections in humans and animals.
Antibiotic growth promoters (AGPs)	Antibiotic agents historically used in subtherapeutic doses to prevent disease and enhance production performance in food animals.
Ionophorous anticoccidials	Agents with antiparasitic (anticoccidial) and antibacterial effects (e.g. narasin). Also known as polyether ionophores or polyether antibiotics.
Synthetic anticoccidials	Agents with antiparasitic (anticoccidial) effects. Also known as 'chemicals' or non-ionophore anticoccidials.
Alternatives to antimicrobials (ATAs)	Non-antibiotic feed additives used as substitutes for conventional in-feed antimicrobials (i.e. AGPs and ionophores).

1.2.1 Antibiotic growth promoters

Historically, antibiotic agents have been used not only to treat bacterial infections, but also to prevent disease and promote growth (Page & Gautier, 2012). After the discovery of the performance-enhancing effect of subtherapeutic doses of antibiotics in the late 1940s, these substances collectively termed antibiotic growth promoters (AGPs) were gradually implemented as additives in livestock feed worldwide (Laxminarayan et al., 2013). This practice has undeniably contributed to the spread of antibiotic resistant pathogens. In the late 1960s, the United Kingdom (UK) Government appointed a committee to deliver an evaluation of antibiotic use in agriculture and veterinary medicine. The background was an increasing trend of untreatable bacterial infections in both animals and humans, and a suggested link between AMR and antibiotic use in agriculture. In 1969, the influential 'Swann report' was published (Kirchhelle, 2018b). The report acknowledged that the use of antibiotics in agriculture contributed to a rise in development of AMR, and recommended that antibiotics used in human medicine should not be permitted as AGPs in animal feed. In the following years, the Swann report led many countries to reconsider the use of antibiotics such as tetracyclines and penicillin on a routine basis in livestock farming (Kirchhelle, 2018a).

Sweden was the first country to ban the use of antibiotics for growth promotion in 1986 (Wierup, 2001). During the next decade, the use of AGPs was abolished in Norway (1995), Denmark (1998-1999) and Finland (1999) (Grave et al., 2006; Laine et al., 2004). National initiatives in several countries led to common European Union (EU) regulations and directives banning the use of specific antibiotics such as avoparcin, spiramycin and virginiamycin in animal feedstuffs due to their similarity with antibiotics used in human medicine (Castanon, 2007). After recommendations from amongst others the World Health Organization (WHO), a final ban of the use of antibiotics other than anticoccidials and histomonostats (i.e. substances primarily used to control intestinal protozoan parasites) as additives in animal feed was implemented in 2006 in the EU. In the United States (US), medically important antibiotics were forbidden to use for growth promoting purposes as from 2017, and the authorities have released guidelines and recommendations which aim to reduce the overall use of antibiotics in livestock (FDA, 2013; Noack et al., 2019).

In developing countries, antibiotics are often abused or misused due to availability, non-compliance or lack of regulations and education (Ayukekbong et al., 2017). The increased demand for animal protein in these countries has promoted more intensive farming systems, and antimicrobials of various classes are sometimes used as low-cost substitutes for more expensive hygiene and biosecurity measures. Low socioeconomic status and poor hygienic conditions make reduction of antimicrobial use in developing countries more complex and difficult than in developed countries.

1.2.2 Ionophorous anticoccidials

The worldwide focus on reducing the use of antibiotics in the broiler industry is substantial (EIP-AGRI, 2021; Mehdi et al., 2018). Less attention has been spent on the use of ionophorous anticoccidials, also designated polyether antibiotics or ionophores, which are considered the most extensively used drugs in broiler production as of today (Noack et al., 2019). Ionophores are primarily used as a prophylactic measure against coccidiosis in broilers, and have been used in many countries since the 1970s (Chapman et al., 2010). Coccidiosis in broiler chickens is caused by protozoan parasites belonging to the genus *Eimeria*. After ingestion of sporulated oocysts, the parasites replicate intracellularly in intestinal epithelial cells of the host and often cause extensive damage to the intestinal mucosa when they break out of the cells (Gerhold, 2016). Pathogenicity is influenced by many factors, e.g. diet, *Eimeria* species, number of oocysts ingested, age and health status of the host. Milder infections can be subclinical, or may cause loss of appetite, depression and weight loss. Symptoms of severe coccidiosis include diarrhoea and increased flock mortality. Ionophores prevent coccidiosis by affecting the passage of ions across parasite cell membranes, resulting in non-physiologic ion gradients that arrest parasite development or cause cell death (Chapman, 1997).

Damage in the intestinal lining with subsequent leakage of proteins and reduced absorptive capacity make the chickens more susceptible for secondary infections (Van Immerseel et al., 2004). Coccidiosis predisposes for *Clostridium perfringens*-associated necrotic enteritis, which is one of the most economically important bacterial infections in broiler production. Necrotic enteritis is a widespread intestinal disease that causes substantial production losses, impaired animal welfare and in severe cases, high mortality rates (Timbermont et al., 2011). The Gram-positive bacterium *C. perfringens* is

an intestinal commensal, i.e. it is commonly found in the intestines of chickens without causing any illness. However, under certain conditions such as increased access to substrates and essential amino acids, bacteria will start proliferating and produce harmful, pore-forming toxins which damage the small intestinal mucosa (Keyburn et al., 2010a). The impact of infection with *Eimeria* spp. on proliferation of *C. perfringens* with subsequent development of necrotic enteritis is illustrated in Figure 2.

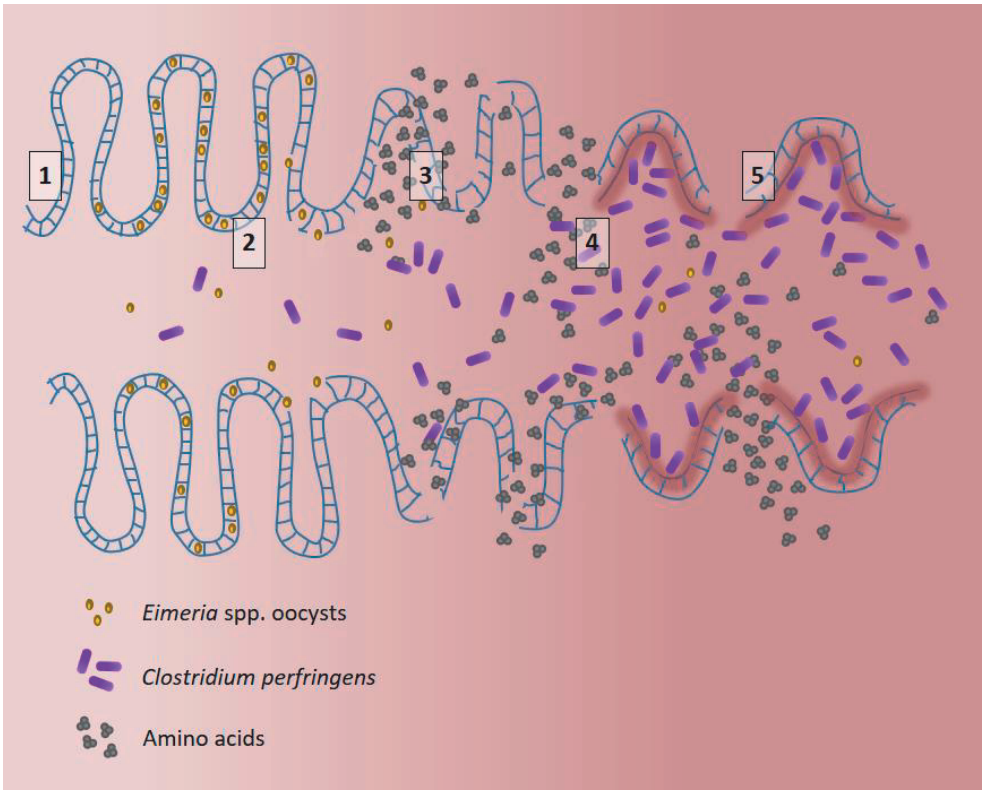

Figure 2. Development of necrotic enteritis. 1. Healthy intestinal mucosa. 2. Infection with *Eimeria* spp. cause disruption of intestinal epithelial cells when oocysts break out of the cells. 3. Damaged intestinal mucosa results in increased mucus production and leakage of amino acids into the intestinal lumen. 4. Increased access to appropriate substrates provides a growth advantage for *C. perfringens*, leading to rapid bacterial proliferation and colonization of the small intestine. 5. Pore-forming and other toxins produced by *C. perfringens* induce further damage to the intestinal mucosa, and the chickens may develop necrotic enteritis. Illustrated by S. Granstad (2021).

Ionophores such as narasin possess both anticoccidial and antibacterial properties, and are considered to have a prophylactic effect against *C. perfringens*-associated necrotic enteritis (Brennan et al., 2001; Lanckriet et al., 2010). According to Regulation (EC) No. 1831/2003, ionophores are classified as feed additives and do not require veterinary prescription (European Parliament and Council, 2003). Hence, such agents were not affected by the EU ban in 2006, regardless of their antibiotic effect. Removal of AGPs has led to increased importance of ionophores in many countries (Grave et al., 2004; Wierup, 2001).

Use of ionophores over time promotes the emergence of *Eimeria* strains resistant to these substances (Chapman, 1997). Development of resistance to one ionophore can also make the coccidia resistant to other ionophores ('cross-resistance'). Resistance against ionophores has also been found in intestinal bacteria in broilers (Butaye et al., 2000). According to the surveillance programme Norwegian Veterinary Antimicrobial Resistance Monitoring (NORM-VET), as much as 50-80% of tested broiler flocks in Norway in the period 2002-2013 harboured faecal narasin-resistant bacteria (VKM, 2015).

The use of ionophores in livestock industries has generally been considered safe with regard to AMR since this chemical class of drugs is not used in human medicine. However, in 2012, a group of researchers hypothesised that the use of narasin might be associated with persistence of vancomycin resistance in intestinal bacteria from broilers (Nilsson et al., 2012). This report contributed to considerable concerns regarding the routine use of ionophores in broiler feed, especially in Norway (VKM, 2015). More recently, a study demonstrating a significant decrease in vancomycin-resistant intestinal bacteria in Swedish broilers concomitantly with an increased use of narasin was published (Nilsson et al., 2019). Although this study weakened the hypothesis on an association between selective pressure caused by ionophores and persistence of AMR to antibiotics important in human medicine, the role of ionophores in this context remains uncertain (Naemi et al., 2020). Mechanisms of ionophore resistance are not well understood, and the existing evidence base is not considered sufficient to rule out the possibility that ionophores contribute to bacterial resistance against antibiotics of therapeutic importance in human medicine (O'Neill, 2015; Wong, 2019).

1.3 Prevention of intestinal disease without in-feed antimicrobials

Over the past decades, there has been an increased focus on alternatives to antimicrobials for prevention and control of necrotic enteritis in broilers (Caly et al., 2015; Dahiya et al., 2006; M'Sadeq et al., 2015). Nutrients, microorganisms, non-digestible substrates, antimicrobials, toxins, toxic substances, the immune system and the mucosal barrier are some of the many factors that influence the 'intestinal homeostasis', and disruption of the balance between these elements could result in 'dysbiosis' (Das & Nair, 2019). Intestinal homeostasis may be defined as a well-functioning balance between all components in the gut environment. Dysbiosis is a term used for complex and not clearly defined intestinal conditions which arise as a result of compositional and/or functional imbalance in the ecological community of microorganisms ('microbiota'). Development of necrotic enteritis is believed to be initiated by factors that change the balance of the intestinal microbiota and favour proliferation of pathogenic *C. perfringens* strains (Antonissen et al., 2016; Moore, 2016). Diet, biosecurity and host immunization are some of the key factors in maintenance of intestinal homeostasis and prevention of intestinal disease in antimicrobial-free broiler rearing.

1.3.1 Nutritional strategies

Basic ingredients in broiler diets are cereal grains, cereal by-products, fats, protein meals, amino acids, vitamins and minerals (FAO, 2021). In addition, enzymes, antimicrobials and other dietary supplements are sometimes incorporated in the diet. Both physical and chemical characteristics of the diet ingredients affect gut health, and dietary components are believed to play an important role in development of necrotic enteritis in broilers (Choct, 2009; Yegani & Korver, 2008). Non-digestible ingredients and nutrients that escape digestion and absorption in the upper gut could serve as substrates for microorganisms in the hindgut, and thus influence modulation of the intestinal microbiota and formation of microbial metabolites with beneficial or adverse health effects (Pan & Yu, 2014; Rinttila & Apajalahti, 2013).

Feed composition varies between countries and continents depending on availability and price of raw materials. Some of the most commonly used cereal grains in broiler diets are maize, wheat, barley and rye (Iji et al., 2019). Cereal grains are mainly added to

provide energy, and the major energy-providing carbohydrate in these grains is starch. Wheat, barley and rye have been identified as risk factors for necrotic enteritis in broilers in several studies (Annett et al., 2002; Kaldhusdal & Skjerve, 1996; Riddell & Kong, 1992). The association between cereal grains and increased incidence of necrotic enteritis might be explained by the ability of *C. perfringens* to degrade starch (Groves & Grounlund, 1969; Shih & Labbe, 1996). Events causing incomplete nutrient digestion and absorption in the host (e.g. loss of absorptive surface due to coccidiosis or increased digesta viscosity due to high levels of non-starch polysaccharides in grains) could possibly cause favourable growth conditions for *C. perfringens* and other microorganisms of the resident microbiota able to utilize starch (Figure 3).

Other dietary ingredients together with physical or chemical characteristics of feedstuffs have been proposed as predisposing factors for development of necrotic enteritis in broilers. It has been suggested that dietary fat source could influence proliferation of *C. perfringens*, as diets with animal fat (lard and tallow) resulted in higher intestinal *C. perfringens* counts compared with a similar diet with vegetable oil as the source of fat (Knarreborg et al., 2002). Additional examples of possible dietary necrotic enteritis predisposing factors are high levels of animal protein (particularly fishmeal), non-starch polysaccharides, feed particle size and mycotoxins (Antonissen et al., 2014; Engberg et al., 2002; Prescott et al., 2016; Timbermont et al., 2011). Nutritional strategies for prevention of necrotic enteritis in broilers need to address these disease-predisposing factors. Also, beneficial effects of dietary components on intestinal health should be considered. More knowledge of feed constituents that create unfavourable growth conditions for *C. perfringens*, possibly affect the genotype composition of the *C. perfringens* population or the expression of toxins, or reduce the host susceptibility to disease, would be advantageous.

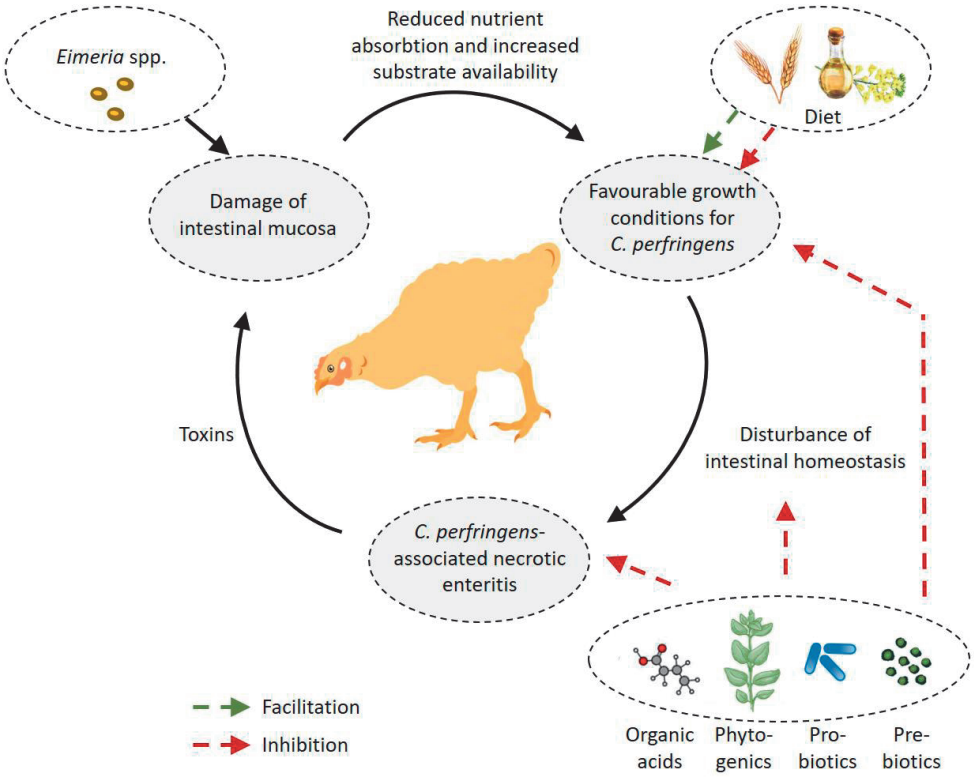

Figure 3. The impact of coccidiosis (*Eimeria* spp.), dietary factors and non-antibiotic feed additives in development and prevention of *Clostridium perfringens*-associated necrotic enteritis in broiler chickens. Some dietary factors have been identified as predisposing factors for necrotic enteritis (indicated by green dotted arrow). Other dietary ingredients and non-antibiotic feed additives have been proposed as preventive measures that may reduce the risk of intestinal disease (indicated by red dotted arrows). Illustrated by S. Granstad (2021), with modified elements from https://www.dreamstime.com, images 120237702/143603723/197287411/110153740.

1.3.2 Feed additives

The use of feed additives as substitutes for antimicrobials in the broiler industry is a trending research topic (Mehdi et al., 2018; Suresh et al., 2018). Such feed additives, sometimes designated alternatives to antimicrobials (ATAs), can be of natural or synthetic origin. A common trait for commercially available feed additives is that they are marketed to be beneficial for intestinal health and productivity. Examples are

probiotics, prebiotics, organic acid-based products, plant-derived products and various combinations of these (Figure 3).

Prebiotics are non-digestible components of the feed (e.g. oligosaccharides) that provide health benefits to the host through stimulation of growth or activity of bacteria present in the intestine (Gibson & Roberfroid, 1995). Undigested prebiotic components are metabolized into microbial fermentation products such as short-chain fatty acids (SCFAs) and lactic acid, and could serve as a barrier for pathogen colonization by acting as a selective nutrient source for beneficial, non-pathogenic bacteria (Ricke et al., 2020). Suggested mechanisms of prebiotics are provision of substrates, prevention of pathogen adhesion, modification of inflammatory responses and preservation of the gut barrier integrity and function through interactions with the intestinal microbiota (Pourabedin & Zhao, 2015).

Probiotics are live microorganisms which provide health benefits to the host when administered in adequate amounts (FAO & WHO, 2002). The probably best-known characteristic of probiotics are their ability to compete for adhesion to host mucosal receptors with pathogenic microorganisms, known as the principle of 'competitive exclusion' (van Zyl et al., 2020). Commonly recognised mechanisms of action among a diversity of probiotic strains are gut barrier reinforcement, competition for available nutrients and production of useful metabolites, enzymes or substances with antimicrobial activities (Hill et al., 2014).

Synbiotics are defined as combinations of live microorganisms (probiotics) and substrates selectively utilized by host microorganisms (prebiotics) that functions cooperatively in provision of health benefits to the host (Swanson et al., 2020). The synergistic synbiotic approach has the advantage of potentially increasing the success of probiotic microorganisms since appropriate substrates are available.

The broad group of phytogenic feed additives comprises a wide selection of bioactive compounds mainly derived from plant parts, herbs and spices (Windisch et al., 2008). The heterogeneity of this group of feed additives makes it difficult to characterize common mechanisms of action. Antioxidative, immunomodulatory and antimicrobial

effects, along with morphological and physiological effects on the intestine, are suggested (Suresh et al., 2018). Some phytogenic feed additives affect feed palatability and could thus influence feed intake (Yang et al., 2015). Essential oils (i.e. volatile lipopholic substances), oleoresins (i.e. extracts composed of resin and oil derived from non-aqueous solvents) and derivatives from these categories of phytogenics have shown potential for prevention of necrotic enteritis and augmentation of performance in broilers (Gadde et al., 2017; Kettunen et al., 2015; Lee et al., 2013; Timbermont et al., 2010).

Organic acids used as feed additives are typically monocarboxylic acids of various lengths, designated as short-chain fatty acids (e.g. formic, acetic, propionic and butyric acid) or medium-chain fatty acids (e.g. lauric acid) (Khan & Iqbal, 2016). Differences in physical and chemical properties of organic acids are believed to affect their modes of action. Some organic acids are believed to exert antibacterial activities by killing bacteria directly through cell-wall penetration or indirectly by pH modification favouring acid-tolerant bacterial species in the gastrointestinal tract (Gadde et al., 2017; Huyghebaert et al., 2011).

In addition to the mentioned categories of feed additives, various enzymes, bacteriophages, bacteriocins, clay minerals, metals and other substances are suggested as disease-preventing and performance-stimulating alternatives to antibiotics (Caly et al., 2015; Gadde et al., 2017). The majority of existing studies of feed additives as alternatives to in-feed antimicrobials evaluate effects of only one or a few feed additives, often within the same feed additive category. These studies may differ considerably in study design and quality, making it questionable to compare the practical value of different feed additives based on test results obtained from different studies. Consequently, more studies with uniform and standardized testing conditions would be valuable. Also, evaluations of a wider variety of feed additives within each study would possibly reveal whether feed additives with different characteristics are more effective in specific experimental models or in distinct rearing phases. Finally, studies with an experimental model representative of the broiler rearing system of interest would increase the relevance of the obtained results.

1.3.3 Management and biosecurity

A group of experts from a European Innovation Partnership (EIP-AGRI) listed biosecurity as the historically most effective strategy among practices for reducing antimicrobial use in poultry production (EIP-AGRI, 2021). External biosecurity, i.e. measures that prevent external infectious agents from entering the farm, was considered most important, followed by general cleaning, disinfection and hygienic standard of feed and water.

Since coccidiosis is identified as an important predisposing factor for necrotic enteritis in broilers, prevention of *Eimeria* spp. infection would be an important strategic measure to avoid development of necrotic enteritis. Sporulated *Eimeria* spp. oocysts are very resilient and can survive in the environment for a long period of time (Gerhold, 2016). The tremendous reproduction capabilities of coccidia contribute to high contamination levels in farm buildings, and the oocysts can resist commonly used disinfectants. Consequently, strict biosecurity leading to *Eimeria*-free broiler houses is almost impossible to attain in practice, but cleaning, disinfection and maximizing the downtime period between flocks can significantly reduce the number of parasites in chicken houses (Chapman et al., 2016; Peek & Landman, 2011). Moreover, strains of *C. perfringens* are ubiquitous in broiler farms. The bacteria form spores that are particularly challenging to eradicate (Talukdar et al., 2017). However, biosecurity and management measures could decrease the total load of bacteria, which would reduce the threat from opportunistic pathogenic *C. perfringens* strains. The majority of remaining *Eimeria* spp. oocysts and *C. perfringens* spores are removed when the litter is cleared from the house, which strongly advocates for litter replacement between successive flocks.

Management and environmental factors associated with feed, water, bedding, stocking density, temperature, humidity, lighting and ventilation are important to maintain health and welfare in broiler flocks. Factors that cause stress could suppress the immune system and increase the risk of necrotic enteritis (Tsiouris, 2016).

Biosecurity and management measures alone are considered insufficient to prevent coccidiosis and necrotic enteritis in chickens raised on litter since both *Eimeria* spp. and

C. perfringens are very difficult to eradicate, and are therefore more or less ubiquitous in the environment of intensive broiler rearing systems. However, such measures reduce the exposure to these infectious agents and are thus of additional strategic value together with other disease preventive strategies.

1.3.4 Vaccination

There are several commercial anticoccidial vaccines consisting of live sporulated oocysts (attenuated or non-attenuated) available for use in broilers. The *Eimeria* strains in attenuated vaccines are manipulated so that their virulence is decreased, or so that the number of oocysts produced during infection is reduced ('precocious lines') (Peek & Landman, 2011). The latter is often considered the preferred option, since the risk of clinical disease is minimized without reduction of immunogenicity (Shirley et al., 2007). Live anticoccidial vaccines can be administered to day-old chicks at the hatchery via spraying, or via drinking water, edible gels or on the feed (Chapman et al., 2002; Soutter et al., 2020).

There are several challenging aspects with vaccination against coccidia. The vaccine production process is costly and logistically demanding, involving infection of live chickens and formulation of oocyst blends from multiple *Eimeria* species. Oocysts lose their infectivity over time, affecting the shelf-life of the vaccines. The methods of administration can make it challenging to ensure that all chickens receive the correct dose. Dosage errors may result in insufficient immune response or asynchronous development of immunity in the flock. If the vaccine dose is too high, significant damage to the intestinal epithelium and clinical coccidiosis can occur, especially if non-attenuated vaccines are used. Since uniform vaccine administration is not always accomplished, reinfection by oocyst from the litter is required for development of robust immunity within the flock (Soutter et al., 2020). New and effective vaccine candidates are needed, and for cost effectiveness and welfare reasons it is preferable that these vaccines do not require live chickens in the production process. Novel recombinant vaccines directed against relevant antigens from multiple *Eimeria* species appear as a technically attractive alternative, but it has proven difficult to identify candidate immunogens that are capable of stimulating protective immune responses in the large

and complex pool of potential *Eimeria* spp. antigens (Shirley et al., 2007; Soutter et al., 2020).

Even though coccidiosis is recognized as an important predisposing factor for necrotic enteritis, it is not essential for development of disease, as necrotic enteritis have been successfully produced without the use of *Eimeria* spp. (Cooper & Songer, 2010; Moore, 2016). This emphasizes that an effective vaccine against necrotic enteritis is needed. Several attempts to make vaccines against necrotic enteritis based on various targets and technologies have been made (Mot et al., 2014). These vaccines have provided varying degrees of protection, but the major challenge has been to develop an effective vaccine that is safe, affordable and compatible with general management practices in broiler industry (Rood et al., 2016). If efficient, practical and affordable necrotic enteritis vaccines were available, these would probably be the most effective alternatives to in-feed antimicrobials for protection against this disease in broilers. Regardless of the mentioned challenges with anticoccidial vaccines, they are viable options for reducing the predisposing potential of *Eimeria* spp. infections in rearing of broiler chickens without ionophores.

1.3.5 Other alternatives

Synthetic non-ionophore anticoccidials ('chemicals') can be used as alternatives to ionophores for prevention of coccidiosis (Peek & Landman, 2011). These compounds have other modes of action compared to ionophores, including inhibition of different biochemical pathways of the parasite metabolism (Noack et al., 2019). However, rapid acquisition of parasitic drug resistance and regulatory limitations exclude synthetic anticoccidials as an option for coccidiosis control in many countries.

Novel and rapid on-farm tests used for surveillance and diagnostics have the potential to provide early detection of infections and make it possible to initiate action before the disease progresses (EIP-AGRI, 2021). Precision livestock farming and data-driven decision-making are concepts in which all relevant data associated with a flock is gathered and analysed. Exchange of information between different parts of the production chain, collection of data over time and experience-based learning can be used to identify farm-specific risk factors, suggest effective prophylactic measures and

anticipate the need for antimicrobials within individual flocks. Consequently, technological solutions could play a key role in broiler production systems without routine administration of in-feed antimicrobials.

1.4 Antimicrobial-free broiler production

The use of AGPs is banned in many countries, and there is an ongoing global awareness on reducing the use of therapeutic antibiotics in the broiler industry (Roth et al., 2019). Ionophores are, however, still extensively used in many parts of the world, including the EU and the US (Noack et al., 2019). In Report COM/2008/0233, the European Commission recommended to maintain the current legislations, allowing the use of ionophores as feed additives due to a lack of effective alternatives (European Commission, 2008). The abolition of ionophores as feed-additives was considered to affect animal health and welfare negatively, and to compromise severely the economy in modern broiler production in the EU. Furthermore, the World Organisation for Animal Health (OIE) has described ionophores as 'essential for animal health' and 'critically important in poultry' in view of their importance in controlling coccidiosis (OIE, 2019).

1.4.1 Situation in Norway

Routine ionophore feed supplementation has been practiced in conventional rearing of broilers in Norway since 1988, and the most predominantly used anticoccidial agent in the Norwegian broiler chicken industry has been narasin (1995-2015) (Haug et al., 2008; VKM, 2015). As in many other countries worldwide, production and consumption volumes of broiler meat has increased steadily in Norway for several decades (Knutsen, 2020). However, in the years 2014 and 2015, the consumption and sale of broiler meat dropped substantially, which resulted in overproduction, price fall and reduction of production volumes (Figure 4)(Rye et al., 2019). The background for the sudden and sharp decline in broiler meat demand was a considerable public and media focus on possible negative consequences of using narasin as a feed-supplement. Reports from NORM-VET confirmed that bacteria resistant to antibiotics defined as critically important in human medicine were occasionally found in samples from broilers in Norway (NORM-VET, 2015). Assumptions of possible cross-resistance and persistence of AMR associated with the use of narasin contributed to a pronounced rise in consumer

demand for chickens reared without any antimicrobials. Consequently, the use of narasin as a feed additive was gradually phased out in the Norwegian broiler industry in the period February 2015 to June 2016 (NORM-VET, 2017). Concerns related to animal welfare, risk factors for disease, use of therapeutic antibiotics, feed utilization and farmer economy were raised in the process, and more knowledge of compensatory measures was requested.

Figure 4. Meat production measured in tonnes in Norway in the period 2002 - 2018. *Other includes meat from various types of poultry other than broiler chickens (ostrich, quail, duck, goose, turkey, hen and cockerel), horses and game. Modified from Rye et al. (2019).

1.4.2 Future outlooks and trends

For the time being, ionophores are not used for any purpose in human medicine (Noack et al., 2019). Consequently, these feed additives are not included in the WHO list of critically important antimicrobials for human medicine (WHO, 2019). However, studies have suggested ionophores as potential chemotherapeutic agents for cancer treatment and as novel drugs in treatment of multidrug-resistant bacterial infections in humans (Huczynski, 2012; Kaushik et al., 2018; Kevin et al., 2009). In veterinary medicine, ionophores are suggested as substitutes for antibiotics in treatment of common bacterial infections (Hickey et al., 2018). Repurposing the use of ionophores in human and

veterinary medicine would put pressure on the global broiler industry to phase out ionophores as a prophylactic measure.

General public concerns related to AMR and antibiotic residues in animal products worldwide have paved the way for chicken meat products labelled 'no antibiotics' and 'raised without antibiotics', meaning that no antibiotics or ionophores are used from hatch to slaughter (Cervantes, 2015). Together with potentially unknown risks of AMR associated with the use of ionophores, the market trends and increased consumer awareness imply that the routine use of ionophores eventually would need to be reconsidered in many countries.

International standards and guidelines for the use of antimicrobials in veterinary medicine and livestock industries are established to promote proper use of antibiotics (FAO et al., 2020). One of these standards, 'Prudent and efficient use of antimicrobials in pigs and poultry', encourages rational use of antibiotics in broiler flocks without mentioning ionophores specifically (Magnusson et al., 2019). General recommended principles are to avoid regular preventive use of antibiotics and to use antibiotics only after diagnosis of disease from a veterinarian. The Federation of Veterinarians of Europe (FVE) has advocated for increased veterinary oversight of ionophores through required veterinary prescription of these agents (FVE, 2016). Better surveillance, correct use and compliance with withdrawal periods were some of the key arguments behind the position. Improved control and monitoring of ionophore usage could possibly be an effective first step towards a gradual reduction and removal of ionophores as a prophylactic measure in the global broiler industry.

Coccidiosis and necrotic enteritis are some of the most important production-limiting diseases in broilers (Blake et al., 2020; Wade & Keyburn, 2015). A possible abolition of the prophylactic use of ionophores in the global broiler industry calls for more research on alternative measures for disease prevention. Dietary factors are known to have a significant impact on gastrointestinal health. Critical evaluations of disease-preventive or disease-predisposing effects of feed ingredients could be used to define ideal feed compositions in broiler rearing without AGPs or ionophores (Figure 5). More knowledge of feed ingredients and additives that contribute to animal health, welfare and

productivity is requested to achieve sustainable antimicrobial-free broiler production systems worldwide.

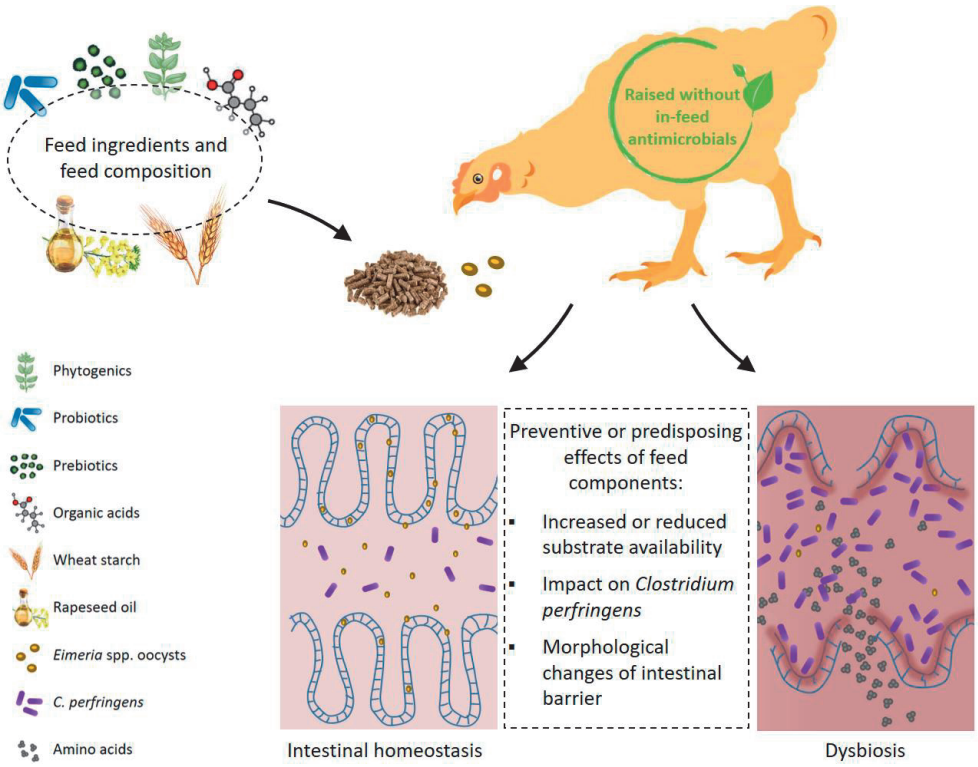

Figure 5. Possible preventive or predisposing effects of feed components on development of *Clostridium perfringens*-associated necrotic enteritis in broiler chickens reared without in-feed antimicrobials (AGPs and ionophores). Feed ingredients could cause increased or reduced availability of preferred substrates for beneficial or pathogenic microbes, or have direct or indirect effects on *C. perfringens* colonization and proliferation. Furthermore, components of the feed could cause modulations of intestinal morphology and thereby strengthen or weaken the intestinal barrier. Illustrated by S. Granstad (2021), with modified elements from https://www.dreamstime.com, images 120237702/143603723/197287411/ 110153740.

2. Aims and objectives

The overall aim of this thesis was to study effects of dietary manipulations in broiler chickens reared without in-feed polyether ionophores, antibiotic growth promoters and therapeutic antibiotics, and thereby contribute to increased knowledge of the interplay between feed composition, intestinal health and productivity in antimicrobial-free broiler production systems.

The research objectives were to:

1. Test a representative selection of commercially available non-antibiotic feed additives and some combinations of these feed additives with claimed beneficial impact on intestinal health and/or physiology in an experimental animal model simulating commercial broiler house conditions. Further, use the obtained results to identify promising active components with preventive effect against *C. perfringens*-associated necrotic enteritis and beneficial effect on production performance (Paper I).

2. Study the impact of dietary starch to fat ratios on the dynamic development of digestibility, digestive enzymatic activity, production performance, intestinal morphology, *C. perfringens* counts and toxin profile, necrotic enteritis prevalence and short-chain fatty acid concentrations in *Eimeria*-challenged broilers (Paper II and III).

3. Summary of papers

3.1 Paper I

Effect of feed additives as alternatives to in-feed antimicrobials on production performance and intestinal *Clostridium perfringens* counts in broiler chickens

In this paper, non-antibiotic commercially available feed additives were assessed for their effect on production performance and intestinal *C. perfringens* counts in broiler chickens. The study was based on *in vivo* feeding trials employing a uniform experimental model with *Eimeria*-challenge to resemble commercial flock settings. In total, 24 non-antibiotic treatments with active components belonging to the product classes probiotics, prebiotics, phytogenics and/or organic acids and one polyether ionophore treatment (narasin) were compared against a negative control diet without any feed additives. The feed additive treatments were administered to Ross 308 broilers from day 0 to 28. Analyses of production performance and caecal *C. perfringens* counts were based on 11 replicate pens and 33 chickens per treatment, respectively. Chickens receiving feed additives classified as probiotics and prebiotics demonstrated the most effective feed conversion during *Eimeria*-challenge. The plant-based feed additives designated phytogenics improved overall feed conversion and reduced *C. perfringens* counts, whereas the additives based on organic acids increased weight gain independent of age. Two individual non-antibiotic treatments achieved a combined significant reduction of *C. perfringens* counts and improvement of feed conversion ratio during the time span subsequent to *Eimeria*-challenge. One of these treatments was a probiotic based on a strain of the bacterium *Bacillus subtilis*, and the other treatment was a combination of components from the yeast *Saccharomyces cerevisiae*, short- and medium-chain fatty acids and a phenolic compound. However, the beneficial impacts were inferior compared with the effect of the polyether ionophore narasin. In conclusion, the individual non-antibiotic feed additives and different product classes demonstrated beneficial impacts during distinct rearing phases and on specific performance targets. The results from this study can be used to select promising active components and product combinations for future studies. Collectively, the group of non-antibiotic feed additives improved gut health and performance compared with the negative control, suggesting that such feed additives can be useful in modern broiler production systems without use of in-feed antimicrobial agents.

3.2 Paper II

Varying starch to fat ratios in pelleted diets: I. Effects on nutrient digestibility and production performance in *Eimeria*–challenged broiler chickens

This study aimed to investigate the effect of dietary starch to fat ratio on digestibility and production performance in chickens fed antimicrobial-free diets. The hypothesis was that a diet with a high starch to fat ratio (HS) may impair nutrient digestibility and growth performance compared with a diet with a low starch to fat ratio (LS) in *Eimeria*–challenged broilers. From days 10 to 29, chickens in 12 replicate pens were given isocaloric and isonitrogenous steam-pelleted diets with either HS or LS, by replacing the wheat starch in one diet with a mixture of rapeseed oil and sand (inert material) in the other. On day 17, a 10-fold dose of live vaccine strains of *Eimeria* species was administered via drinking water. Ileal samples were collected on days 16 and 29. Analyses revealed that starch content in the ileum tended to be higher on day 16 and was significantly higher on day 29 in the HS group. However, the HS diet did not induce exceedingly high levels of starch in the ileum, suggesting that there was no considerable starch overload in the gut. Ileal starch digestibility was improved with increasing dietary starch level from 23% to 45%. This demonstrated the capacity of *Eimeria*-challenged broiler chickens to digest high levels of starch. Ileal energy digestibility was not affected by the treatments. Weight gain did not differ between treatments; however, chickens fed the LS diet were less efficient in feed conversion compared with those fed the HS diet. The use of isolated starch and the unintended higher extent of starch gelatinization in the HS diet may have contributed to the higher starch digestibility in chickens fed the HS diet. The hypothesis that high ratios of starch to fat in pelleted diets may impair starch digestibility and production performance in *Eimeria*-challenged broiler chickens was not verified. Further work is required to clarify this research question, taking into consideration the physical form of starch source and the potentially confounding role of feed processing on starch availability.

3.3 Paper III

Varying starch to fat ratios in pelleted diets: II. Effects on intestinal histomorphometry, *Clostridium perfringens* and short-chain fatty acids in *Eimeria*-challenged broiler chickens

In this study, the effect of two diets with different dietary starch to fat ratios on intestinal morphology, *C. perfringens* counts and toxin profile, necrotic enteritis prevalence and caecal short-chain fatty acid (SCFA) abundances in *Eimeria*-challenged broilers fed antimicrobial-free diets was investigated. The hypothesis behind the study was that a high-starch diet (HS) would lead to impaired gut health compared to a low-starch diet (LS) in broilers predisposed for development of necrotic enteritis. In total, 1,920 one-day-old Ross 308 broiler chickens were fed one of two isocaloric diets formulated either with high (32:1) or low (2:1) starch to fat ratios from day 10 to 29 of age. Each treatment group had 12 pen replicates with 80 broilers per pen. On day 17, the chickens were challenged with *Eimeria* vaccine strains, inducing peak oocyst excretion on days 21-23. Samples were collected on days 16, 21–23 and 29. Whereas villus length increased gradually throughout the study in the HS group, a peak level was reached on days 21–23 in the LS group. On day 29, the HS group had significantly longer villi than the LS group. Caecal SCFA concentrations were higher in the HS group compared with the LS group on day 16. In both groups, the SCFA level peaked on days 21–23, with the most pronounced increase seen in the LS group. Caecal *C. perfringens* counts and necrotic enteritis prevalence were similar in the two groups. A multiplex real-time quantitative PCR assay targeting the toxin-encoding genes *cpa* and *netB* of *C. perfringens* was used to determine the ratio of presumptively pathogenic to total *C. perfringens* (*netB:cpa* ratio) in caecal samples. The *netB:cpa* ratio increased from day 16 to 29 in the HS group, and the overall frequency of *netB*-positive intestinal samples increased from 79% on day 16 to 100% on day 29. In summary, the most important outcomes from this study were that dietary starch to fat ratio affected the dynamics of small intestinal villus length and caecal SCFA abundance in chickens challenged with *Eimeria* spp. These findings suggest that chickens adapt to different dietary starch levels by structural remodelling of the small intestine, and that caecal SCFA abundance is associated with the availability of substrate for the microbiota in the posterior intestinal segments. Chickens adapted to higher levels of dietary starch might be more resilient to *Eimeria* spp. infections due to increased mucosal surface area. Studies with other dietary starch sources are required to clarify the impact of different starch levels on intestinal health in broilers during *Eimeria* spp. exposure.

4. Methodological considerations

The following section contains reflections upon some of the methods used in the PhD study. Detailed descriptions of the materials and methods used are found in the papers.

4.1 Animal experiments

The papers in this thesis are based on *in vivo* experimental feeding trials using Ross 308 broiler chickens. Animal experiments were necessary because *in vitro* alternatives to living chickens were neither available nor applicable at the time. All use of live animals in research must be ethically justifiable. In Norway, animal experiments require advance approval from the Norwegian Food Safety Authority (Mattilsynet), and Directive (EU) 2010/63 on the protection of animals used for scientific purposes (European Parliament and Council, 2010) must be followed. The number of animals used in the experiments were based on experiences from previous similar studies, in which the appropriate sample size for the relevant outcome variables had been determined. The feeding trials were conducted at the research facility Scandinavian Poultry Research with competent personnel caring for the animals. The researchers responsible for the experiments were trained and certified in accordance with the national regulation on the use of animals in research (Forskrift om bruk av dyr i forsøk, 2015).

Metabolic steps, digestive processes, intestinal fermentation processes and the host-associated effects thereof are difficult to study and likely impossible to mimic perfectly *in vitro*. The gut microbiota is a dynamic system with complex relationships between microbial communities and host-related factors such as the immune system (Carrasco et al., 2019). Currently available *in vitro* chicken gastrointestinal models mainly target specific nutrients or digestion processes (Bryan et al., 2018; de Carvalho et al., 2021). These models are unable to simulate the complete process comprising ingestion, digestion, absorption and fermentation, and cannot mirror how the absorbed components of the diet impact the bird outside the gastrointestinal tract. The external validity, i.e. the extent to which research results derived in one setting, population or species can be applied to other settings, populations and species, is presumably high when animal models are used in veterinary research (Pound & Ritskes-Hoitinga, 2018). In the experiments of this thesis, the use of a relevant chicken strain (Ross 308 broilers)

raised in an environment simulating commercial conditions made it possible to study the effect of dietary modulations on digestibility, intestinal pathogens, bacterial fermentation products, intestinal morphology, chicken growth and feed conversion ratio. Furthermore, the use of this *in vivo* model increased the validity of the obtained results for the commercial broiler industry.

A promising strategy for future studies could be to test specific feed ingredients in a two-step manner as described by de Carvalho et al. (2021). Initially, *in vitro* models can be used to study relevant and specific outcomes, such as inhibitory effects on microorganisms (e.g. *Eimeria* spp. and *C. perfringens*), impacts on defined microbial populations or microbial fermentation output (SCFA production). Preliminary data from *in vitro* testing can be used to establish models and guide design for *in vivo* studies. This approach has the potential to ensure properly designed *in vivo* studies and thereby reduce the usage of experimental animals. However, there are still considerable limitations associated with *in vitro* chicken gastrointestinal models, and improvements are needed to increase their relevance in feed studies.

4.2 *Eimeria* challenge model

In order to assess whether feed components could contribute to prevent proliferation of *C. perfringens* and development of necrotic enteritis, a challenge was needed to predispose the chickens for these events. The chosen approach in all experiments of this research was to use *Eimeria* vaccine strains to induce mild to moderate intestinal damage known to favour growth of *C. perfringens* (Figure 2).

An additional purpose behind the challenge was to simulate the conditions in an average Norwegian broiler house. At the research facility used for the feeding trials (Scandinavian Poultry Research) there were strict protocols for management, hygiene and biosecurity. These practices were likely to contribute to an environment with less pathogenic microorganisms and lower infection pressure than in an average commercial broiler house. The *Eimeria*-challenge contributed to create an environment more representative of commercial conditions, and by that increase the practical relevance and external validity of the results obtained from the experiments.

The *Eimeria* spp. oocysts used to challenge the chickens in the feeding trials were obtained from the commercially available anticoccidial vaccine Paracox-5 vet.® (MSD Animal Health). Paracox-5 contains live, sporulated oocysts from five different attenuated lines of *Eimeria* named *E. acervulina* HP, *E. maxima* CP, *E. maxima* MFP, *E. mitis* HP and *E. tenella* HP. Attenuation, i.e. the process of reducing the virulence of the coccidia without killing them, is achieved by selection for precocious lines. This means that the first oocysts that are shed in the beginning of the patent period are selected for further passages, until parasite lines with shorter prepatent period and reduced reproductive capacity are obtained (Peek & Landman, 2011; Soutter et al., 2020). The precocious parasite lines have reduced pathogenicity due to reduced oocyst production, but they are still able to induce some degree of damage to the mucosal lining of the intestine and thus increase the risk of *C. perfringens* proliferation and necrotic enteritis.

During the third week post-hatch, a ten-fold dose of the vaccine was administered to the chickens via the drinking water. The challenge dose was calculated from the number of chickens in each pen (10 x 0.004 ml x 80 chickens). All chickens were deprived of water for at least 2 hours before the vaccine solution was distributed into the drinking system of each pen. Initially, *Eimeria* spp. invade and reproduce within the intestinal epithelial cells, and then the oocysts break out of the cells and cause further damage to the intestinal mucosa. The peak of oocyst excretion after vaccination with live vaccines is expected to occur during the first week after vaccine administration, and more specifically between 96 to 156 hours after vaccination with Paracox-5 (CocciForum Magazine, 2007).

The concept of challenging broilers with *Eimeria* vaccine strains alone is to date a rarely used approach, as most experimental studies on necrotic enteritis includes inoculation with *C. perfringens*. In some studies, inoculation with a ten-fold dose of Paracox-5 or Paracox-8 vaccine has been employed as one of several challenge alternatives (Gholamiandehkordi et al., 2007; Timbermont et al., 2009). Reportedly, the vaccine challenge did not result in any macroscopically visible necrotic enteritis lesions in either of these studies. However, in one of the studies, chickens challenged with the vaccine oocysts demonstrated evidence of gut damage based on various microscopic parameters

in a gut damage scoring system, with total gut damage scores displaying an increasing trend in the period three to six days post challenge (Gholamiandehkordi et al., 2007). In a study evaluating the timing of predisposing factors in necrotic enteritis challenge models, chickens were more likely to have necrotic enteritis lesions when a Paracox-8 overdose was administered four to five days prior to scoring compared with earlier vaccine administration (Van Waeyenberghe et al., 2016). A pilot study was performed to establish the challenge model used in the experiments of this thesis. The pilot study confirmed that *C. perfringens* counts in caecal samples from challenged individuals were significantly higher when compared with unchallenged chickens in the period four to six days after vaccine administration. Consequently, this timespan was regarded a critical window for sampling in all experiments of this research work.

Results from previous studies indicate that *Eimeria* spp. vaccine challenges are relatively mild compared with necrotic enteritis challenge models employing inoculation with *C. perfringens* in combination with *Eimeria*-challenge (Gholamiandehkordi et al., 2007; Timbermont et al., 2009). In the current research work, it was not the intention to study clinical necrotic enteritis, but rather evaluate whether the different feed components of interest could reduce *C. perfringens* proliferation and prevent impaired performance in a commercially representative setting with moderate occurrence of subclinical necrotic enteritis. Based on the ability of the described challenge model to increase caecal *C. perfringens* counts and induce subclinical necrotic enteritis, it was considered an appropriate challenge in the experiments of this thesis.

4.3 *Clostridium perfringens* counts and production performance as measures to study intestinal health

The bacterium *C. perfringens* is an intestinal commensal, meaning that it can be present in the caeca of chickens in moderate numbers without causing disease (Caly et al., 2015). If intestinal homeostasis is disturbed, the growth conditions for *C. perfringens* may improve and lead to intensive bacterial proliferation and colonization of the small intestine. Chickens with necrotic enteritis harbour high numbers of *C. perfringens* in the intestinal tract (>10^5 colony-forming units/g ileal digesta) (Si et al., 2007).

Necrotic enteritis in its clinical form was first described in 1961, and the classical acute form of the disease is characterized by high flock mortality (Parish, 1961; Timbermont et al., 2011). A subclinical form of necrotic enteritis was originally described in 1992 as a mild form of the disease characterized by increased numbers of intestinal *C. perfringens*, characteristic and macroscopically visible lesions in the small intestine, increased feed conversion ratio and impaired growth rate (Kaldhusdal & Hofshagen, 1992). Subclinical necrotic enteritis often remains undiagnosed in commercial flocks. Production losses due to reduced weight gain and increased feed conversion ratio, together with condemnations due to liver lesions (mostly cholangiohepatitis) found during meat inspection at slaughterhouses, can make the negative economic impact of subclinical necrotic enteritis in broiler flocks substantial (Lovland & Kaldhusdal, 1999; Skinner et al., 2010).

A strong statistical association between high caecal *C. perfringens* counts and characteristic small intestinal lesions linked to subclinical necrotic enteritis has been reported (Lovland et al., 2004; Novoa-Garrido et al., 2006). In studies of subclinical necrotic enteritis where macroscopically visible intestinal lesions are rare, enumeration of intestinal *C. perfringens* has been suggested as a possible tool to evaluate the impact of study variables (Gholamiandehkordi et al., 2007). The use of necrotic enteritis prevalence (categorical variable) as an outcome variable in an experimental model where the frequency of intestinal lesions is expected to be low, would require a larger sample size to obtain sufficient statistical power. Based on previous experiences, the challenge used in the experiments of this thesis was expected to cause low prevalence of intestinal lesions. Consequently, continuous outcome variables like caecal *C. perfringens* counts and production performance were chosen as indicators of intestinal health status, on the basis of being considered relevant, efficient and applicable parameters. Suggested associations between subclinical necrotic enteritis, *C. perfringens* counts, production performance and in-feed ionophores and AGPs are explained in Figure 6.

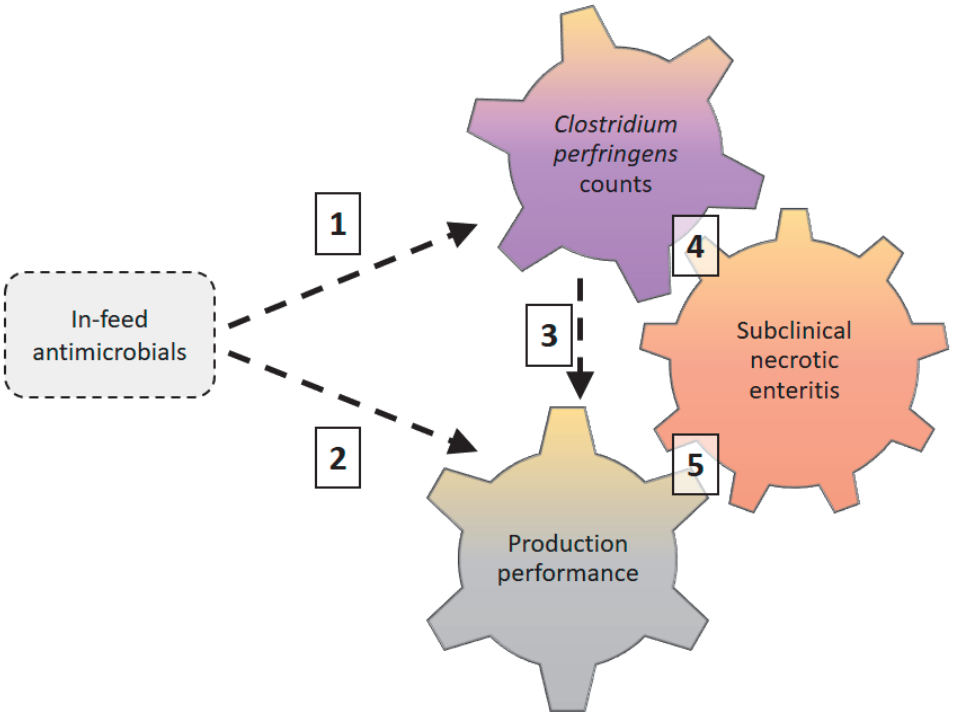

Figure 6. The association between in-feed antimicrobials, intestinal *Clostridium perfringens* counts, subclinical necrotic enteritis and production performance in broiler chickens.

1. Antimicrobials with antibiotic properties (ionophores and AGPs) suppress growth of *C. perfringens in vivo* and *in vitro* (Devriese et al., 1993; Elwinger et al., 1998; Martel et al., 2004; Silva et al., 2009; Stutz et al., 1983; Stutz & Lawton, 1984; Watkins et al., 1997).

2. Ionophores and AGPs are known to have a positive impact on production performance in broilers when administered in appropriate doses (Elwinger et al., 1998; Yan et al., 2021). It is proposed that the 'antibiotic growth effect' is associated with reduction of pathogenic bacteria and other modulations of the microbiota in the intestinal tract (Apajalahti et al., 2004; Dibner & Richards, 2005; Graham et al., 2007; Jacobs et al., 1953; Pedroso et al., 2006).

3. Increased numbers of *C. perfringens* have been associated with reduced performance/growth depression (Lev & Forbes, 1959; Stutz et al., 1983).

4. High intestinal *C. perfringens* counts are considered one of the characteristics of subclinical necrotic enteritis (Cooper & Songer, 2009; Novoa-Garrido et al., 2006; Kaldhusdal & Hofshagen, 1992; Lovland et al., 2004).

5. Subclinical necrotic enteritis is associated with impaired production performance (Kaldhusdal & Hofshagen, 1992; Lovland & Kaldhusdal, 2001; Skinner et al., 2010; Timbermont et al., 2011).

Illustrated by S. Granstad (2021).

There are several methods available for identification and quantification of intestinal microorganisms. Cultivation of bacteria can be described as isolation of bacterial species on selective media (Fraher et al., 2012). It is a well-known and traditional technique for antigenic studies, antibiotic susceptibility testing and identification and description of novel bacterial pathogens (Houpikian & Raoult, 2002). Cultivation of *C. perfringens* has been of special importance as the first step in characterization of bacterial isolates and their toxins. When samples are plated on sheep blood agar plates and incubated in an anaerobic atmosphere, *C. perfringens* usually grow in colonies with a characteristic double zone of haemolysis (Brynestad & Granum, 2002). Plate counting is a method for enumeration of bacteria based on plating of logarithmic dilutions of samples where calculation is based on counting of colonies. The main disadvantages with traditional plating methods for enumeration of bacteria are that they are labour-intensive, requires a lot of reagents and laboratory-space, and that the incubation step needed for colonies to grow is time-consuming compared with more rapid molecular techniques. Today, culture-independent quantification methods such as digital polymerase chain reaction (dPCR) technologies are available, making it possible to measure the absolute copy number of target DNA without using external standards (Dong et al., 2015). One of these digital technologies is based on droplet microfluidics (droplet digital PCR/ddPCR), and has been shown to be an attractive technical platform for detection of pathogens. Optimized ddPCR assays for quantification of bacteria (sometimes referred to as ddCFU) provide viable and precise quantification of bacteria, also in samples with wide dynamic range and high bacterial concentrations (Scheler et al., 2017). Hence, ddCFU is a promising method for quantification of *C. perfringens* in chicken intestinal samples in future studies.

There is growing evidence that, in addition to *C. perfringens*, also other members of the intestinal microbial community are involved in development of necrotic enteritis and influence the performance level of broilers (Antonissen et al., 2016; Carrasco et al., 2019). However, attempts to characterize dysbiotic events and microbial shifts linked to intestinal disease and impaired performance are associated with a high degree of uncertainty. Thus, ddCFU assays supplemented with next-generation high-throughput sequencing technologies represent useful tools in studies of necrotic enteritis, and could

possibly increase our understanding of the interplay between *C. perfringens* and other members of the intestinal microbiota.

4.4 *Clostridium perfringens* toxin genes in intestinal samples

In Paper III, a multiplex real-time quantitative PCR (qPCR) assay targeting the toxin-encoding genes *cpa* and *netB* of *C. perfringens* was used. The purpose of this analysis was to determine the ratio of presumptively pathogenic to total *C. perfringens* (*netB:cpa* ratio) in intestinal samples, as this ratio has been suggested as a faecal marker to monitor subclinical necrotic enteritis in broilers (Goossens et al., 2019). The α-toxin-encoding gene *cpa* is present in all types of *C. perfringens* strains and lies within the chromosome (Petit et al., 1999). The pore-forming toxin named NetB (necrotic enteritis toxin β-like) is encoded by the plasmid-borne *netB* gene, and this toxin is believed to be a key virulence factor associated with necrotic enteritis in chicken (Keyburn et al., 2008).

Studies investigating the presence of the *netB*-gene ('*netB*-prevalence') in *C. perfringens* isolates from chickens with and without necrotic enteritis show variable results (Abildgaard et al., 2010; Drigo et al., 2009; Keyburn et al., 2010b; Martin & Smyth, 2009; Yang et al., 2018). The relatively high *netB*-prevalence in isolates from healthy chickens reported in several studies indicates that the presence of *netB* in *C. perfringens* strains alone is inadequate to predict virulence. The role of predisposing factors in development of necrotic enteritis is well recognized, and it is assumed that such factors are necessary for *netB*-positive *C. perfringens* strains to cause disease (Rood et al., 2016; Smyth & Martin, 2010; Yang et al., 2019).

As discussed previously, the challenge model solely based on *Eimeria* vaccine strains was considered to be mild compared with challenge models combining coccidia and inoculation with *C. perfringens*. Results from Paper III showed that the prevalence of necrotic enteritis measured as percentage of chickens with typical intestinal lesions was relatively low (Figure 7). The experiment was not designed to compare *netB*-prevalence, neither in *C. perfringens* isolates nor in intestinal samples, of chickens with and without necrotic enteritis. However, the results from the multiplex qPCR were used to determine

the proportion of chicken caecal samples in which the *netB*-gene was detected ('*netB*-positive intestinal samples') at three different time points. On day 16, the overall percentage of *netB*-positive intestinal samples from both treatment groups was 79%, which indicates that *C. perfringens* strains with *netB*-carrying plasmids were present in the intestine of the majority of the chickens before *Eimeria*-challenge was introduced (Figure 7). One study reported high *netB*-prevalence in *C. perfringens* isolates from environmental samples taken from a cleaned and disinfected empty broiler house (Engstrom et al., 2012). From this background, it is likely that *netB*-positive *C. perfringens* strains are widespread in the environment of the broiler house prior to placement of chickens, and that these strains together with *netB*-negative *C. perfringens* colonize the intestine of young broilers. Development of clinical disease is dependent on general health status of the flock and presence of predisposing factors.

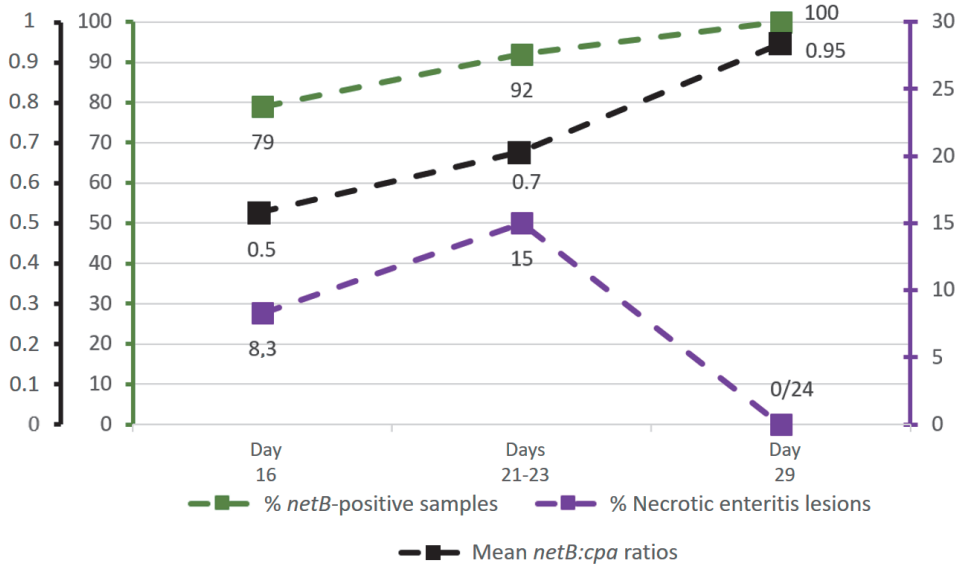

Figure 7. Percentages of *netB*-positive caecal samples, mean *netB:cpa* ratios and prevalence of small intestinal necrotic enteritis lesion in broilers from both treatment groups on days 16, 21-23 and 29. Illustrated by S. Granstad (2021).

The *netB* genes are located on conjugative plasmids, meaning that the toxin genes are parts of extra-chromosomal DNA elements capable of horizontal transmission (Bannam et al., 2011). As reported in Paper III, no necrotic enteritis lesions were detected on day

29, however, all intestinal samples were *netB*-positive at this time point (Figure 7). Strains of *C. perfringens* carrying conjugative toxin plasmids are believed to be able to transfer plasmids to other resident *C. perfringens* strains without these toxin genes (Li et al., 2013). This transfer of plasmids could possibly impact the virulence properties of the recipient strains. The reason for the increase in the proportion of *netB*-positive intestinal samples in Paper III is not known, but possible explanations include dissemination of *netB*-positive *C. perfringens* strains between birds, proliferation of *netB*-positive strains and/or plasmid transfer to *netB*-negative *C. perfringens*.

The *netB:cpa* ratios in intestinal samples did not show any coinciding pattern with necrotic enteritis prevalence at the times of sampling in Paper III. Overall mean *netB:cpa* ratios showed that the proportion of *netB* genes relative to the proportion of *cpa* genes tended to increase in the study period (Figure 7). Studies have shown that *C. perfringens* isolates may carry multiple plasmids (typically one to four), and individual plasmids can carry up to three different toxin genes (Li et al., 2013; Parreira et al., 2012). The complexity of *C. perfringens* plasmid profiles makes the ratio between *netB* and *cpa* genes difficult to interpret. More studies are needed to establish the value of the *netB:cpa* ratio as a marker to monitor subclinical necrotic enteritis in broilers.

5. Results and general discussion

5.1 Non-antibiotic feed additives

The results from Paper I indicate that the effect of alternatives to antibiotics (ATAs) on *C. perfringens* proliferation and production performance vary significantly among different feed additives and within the rearing period. Two rearing phases were emphasised in the study: (1) From day of hatch until two weeks of age, and (2) From two weeks of age until 28 days. The first phase was characterized by unchallenged young broilers with developing digestive systems. In the second rearing phase, the broilers were challenged with coccidia and thus exposed to some degree of intestinal distress. A number of ATAs had beneficial impact on specific variables during distinct rearing phases, suggesting a potential for targeted use of promising active components and combinations of these.

5.1.1 Feed additive classes

When evaluating feed additives from each class as a group, the results demonstrated that phytogenics and probiotics were best able to reduce intestinal *C. perfringens* counts during the critical phase after *Eimeria*-challenge. This phase is presumably characterized by mucosal damage and disturbed intestinal homeostasis, making competitive exclusion by probiotic bacteria or possible antimicrobial effects of phytogenics relevant to impede *C. perfringens* proliferation. The phytogenic class is diverse, and it is unclear whether the beneficial effect on *C. perfringens* counts in this study was a result of antibacterial effects against *C. perfringens*, anticoccidial effects against *Eimeria* spp. or other unknown causal mechanisms. Several reports on anticoccidial effects of different plants, herbs and extracts have been published, and phytogenic feed additives have shown some potential for coccidiosis control in broilers in previous studies (Adhikari et al., 2020; Peek & Landman, 2011).

The probiotic and prebiotic feed additives engendered the most efficient feed conversion in the rearing phase with *Eimeria*-challenge compared with the other non-antibiotic feed additive classes. Since the probiotics also had a beneficial effect on *C. perfringens* counts in the same time period, this class of alternative feed additive shows potential in antimicrobial-free broiler rearing systems. Prebiotic feed additives might

stimulate the beneficial microorganisms already present in the gut, and furthermore increase the survival and success of added probiotic strains ('the synbiotic approach').

The polyether ionophore narasin outperformed the other feed additive classes in weight gain, feed conversion and suppression of *C. perfringens* in both rearing phases, with one exception. The class of organic acid-based feed additives generated similar body weight gain and feed conversion ratio during the two first weeks post-hatch. This finding indicates that organic acids generally promoted growth and effective feed conversion in unchallenged, young broilers in this study. Enhanced performance in young birds might be attractive in the short term, but can possibly be a disadvantage at later rearing stages. Rapid growth is identified as a risk factor for development of cardiovascular and musculoskeletal disorders (Julian, 2005). Increased mortality rate due to such disorders (e.g. right ventricular failure with ascites and sudden death syndrome) severely influence production economy, especially in late rearing stages when these conditions most frequently occur. Furthermore, it has been reported that chickens with the highest body weight and body weight gain prior to *C. perfringens* challenge developed the most severe necrotic enteritis lesions (Dierick et al., 2019). Necrotic enteritis is associated with deteriorated feed utilization and growth, probably due to impaired nutrient uptake by a damaged intestine and reduced feed uptake by sick individuals. Consequently, the advantage of higher growth rate and body weight in young broilers can be lost if the heaviest chickens are more prone to develop severe necrotic enteritis in later rearing stages. Results from Paper I of this thesis showed that the organic acids-based feed additives had no significantly reducing effect on *C. perfringens* counts in the time interval comprising *Eimeria*-challenge. Further studies are necessary to establish whether organic acid-based feed additives could be used in combination with other feed additives to achieve both improved performance in young broilers and protection against intestinal disease in later rearing phases, without increasing the risk for cardiovascular and musculoskeletal disorders.

5.1.2 Active components

In the class of prebiotics, two feed additives (Treatment ID 6/Agrimos® and ID 7/Macrogard®) showed the highest potential for supporting effective feed conversion in the rearing phase comprising *Eimeria*-challenge. Both additives were based on

components and extracts from the yeast *Saccharomyces cerevisiae*. The probiotic feed additive based on the *Bacillus subtilis* PB6 strain (ID 3/Clostat®) was one of the best-performing probiotic feed additives in the study with significantly beneficial effects on both feed conversion ratio and *C. perfringens* counts. Another probiotic based on a *Bacillus subtilis* strain (ID 4/Gallipro®) engendered the most beneficial feed conversion ratio in the time interval with *Eimeria*-challenge. Of the organic acid-based feed additives, the most outstanding product measured in effect on feed conversion ratio was based on sodium formate and formic acid (ID 13/Formi NDF®). Equally strong production performance results in the overall study period were obtained with a product consisting of, among other components, short- and medium-chain fatty acids (including butyric and lauric acid) and a phenolic compound (ID 11/Presan FY®). Feed additives based on the mentioned active components are strong candidates for further testing in commercial broiler production systems.

Among all the ATAs, the lowest *C. perfringens* counts were achieved by a prebiotic based on dehydrated *Saccharomyces cerevisiae* culture with whole cells, metabolites and medium nutrients (ID 16/Diamond V XPC®) and a phytogenic based on oleoresins from turmeric and chilli peppers (ID 21/Xtract Nature®)(Figure 8). However, these additives did not have a significantly beneficial effect on feed conversion ratio in the rearing phase comprising *Eimeria*-challenge, and were consequently not highlighted in Paper I. Some non-antibiotic feed additives may, in similarity with ionophores, affect production performance negatively under certain circumstances or when used in inappropriate doses, irrespective of their impairing effect on *C. perfringens*. Ionophores have a narrow safety margin, and in-feed concentrations marginally higher than recommended doses have been associated with reduced feed intake and depressed growth due to cardiac toxicity, muscle degeneration and neuropathy (Dowling, 1992; Kart & Bilgili, 2008). Non-antibiotic feed additive products with strong reducing effect on *C. perfringens* but with lacking beneficial effect on performance should be tested further for their ability to protect against necrotic enteritis alone or in combination with other feed additives. Effective concentrations, purity of active components and possible safety margins with regard to toxicity are topics of special interest in future studies.

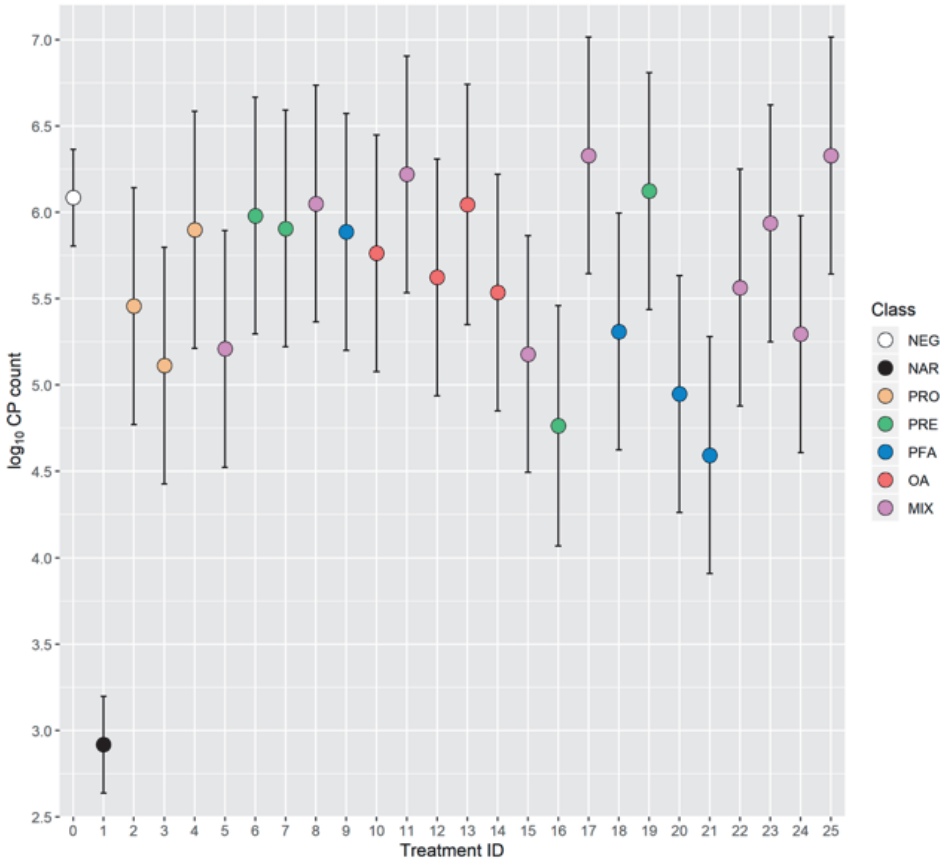

Figure 8. Caecal *Clostridium perfringens* (CP) counts with 95% confidence intervals during the time period four to six days after *Eimeria*-challenge. Negative control (NEG) is treatment 0, narasin (NAR) is treatment 1, probiotics (PRO) are treatments 2–4, prebiotics (PRE) are treatments 6, 7, 16 and 19, phytogenics (PFA) are treatments 9, 18, 20 and 21, organic acids (OA) are treatments 10, 12, 13 and 14, and mixed products (MIX) are treatments 5, 8, 11, 15, 17 and 22-25. Figure retrieved from Paper I.

Two phytogenic feed additives from this study revealed interesting results. A blend of essential oils (ID 9/Digestarom P.E.P. MGE 150®) had significantly beneficial effect on feed conversion ratio, but had no reducing effect on *C. perfringens* counts in the rearing phase with *Eimeria*-challenge. The feed additive based on oleoresins from turmeric and chilli peppers (ID 21/Xtract Nature®) reduced *C. perfringens* counts significantly, but had no beneficial impact on feed conversion ratio in the same period. The association between *C. perfringens* counts and production performance is described in Figure 6.

Similarly, there is growing evidence of an association between other members of the intestinal microbiota and production performance (Johnson et al., 2018; Stanley et al., 2016; Torok et al., 2011). Effects on the host, such as intestinal morphological changes, immunomodulatory effects and altered gut barrier function, are also assumed to influence broiler performance (Carrasco et al., 2019; van der Aar et al., 2017). Thus, ATAs are likely to influence broiler production performance by several possible modes of action (Figure 9). Better understanding of mechanisms behind the performance-enhancing effects and possible negative effects of active components at different dosage intervals, would likely increase the practical value of non-antibiotic feed additives and pave the way for new and improved products.

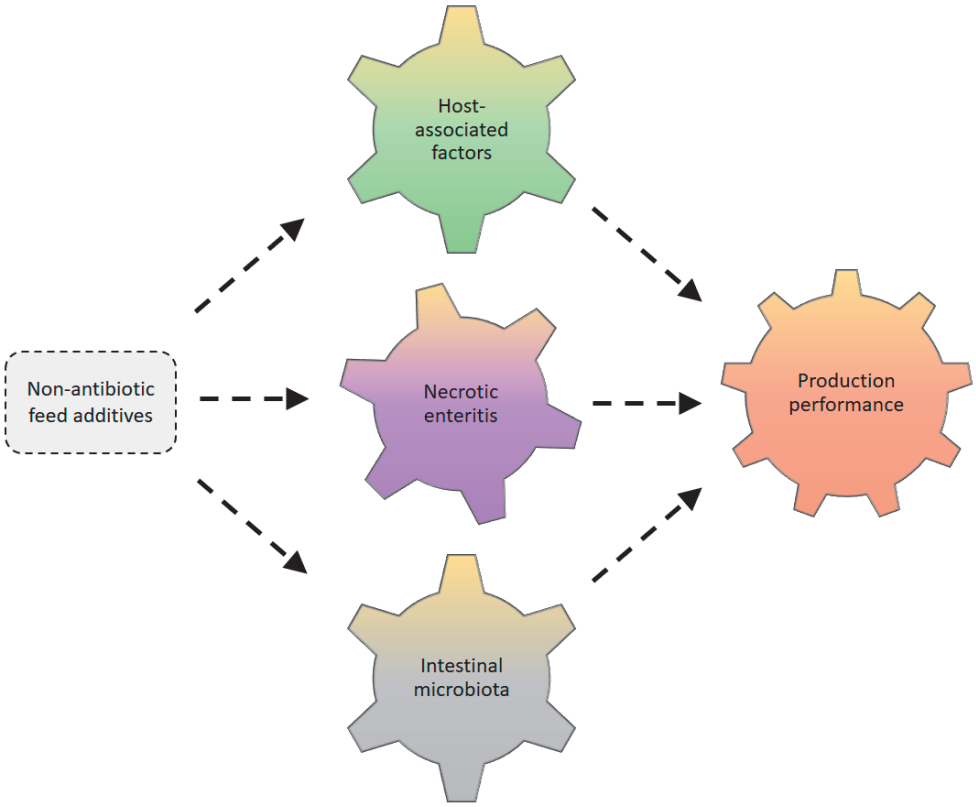

Figure 9. Possible modes of action of non-antibiotic feed additives that may influence production performance include impact on the host (e.g. effects on immune system, gastrointestinal system and metabolism), ability to prevent necrotic enteritis (effects on *C. perfringens* and/or *Eimeria* spp.) and effect on the intestinal microbiota composition. Illustrated by S. Granstad (2021).

Combinations of feed additives with different active components could possibly result in synergistic beneficial effects on intestinal health and productivity. The combination of two feed additives largely based on short- and medium-chain fatty acids, a phenolic compound and components from the yeast *Saccharomyces cerevisiae* (Treatment ID 24) had significant performance-enhancing and *C. perfringens*-reducing effects in the rearing phase with *Eimeria*-challenge in this study. When the two feed additives were tested separately (ID 11/Presan FY® and ID16/ Diamond V XPC®), neither of the two feed additives improved feed conversion ratio in the similar time period. These results imply a synergistic effect of the active components present in the two feed additives in the period with intestinal distress caused by infection with coccidia. However, as demonstrated and discussed in Paper I, the approach of combining two or more feed additives could potentially also diminish the favourable effects of the individual additives. These findings state the need for systematic testing of feed additive combinations in adequate research models.

5.1.3 Targeted use of feed additives

Feed additives with different active components could be used strategically to exploit desired effects for distinct rearing phases. Since feed costs represent a considerable proportion of the overall production costs in broiler farming, a well-founded use of feed additives is required. Results from Paper I could be used to suggest promising active components and targeted use of feed additives based on these components for future testing in commercial broiler flocks. A proposed strategic use of feed additives founded on expected health status in specific age intervals, using a scenario representative of commercial broiler production in Norway, is presented in Figure 10. Effects of the suggested practical solutions and combinations are, however, uncertain. Properly designed studies are needed to establish evidence-based advice for commercial broiler flocks.

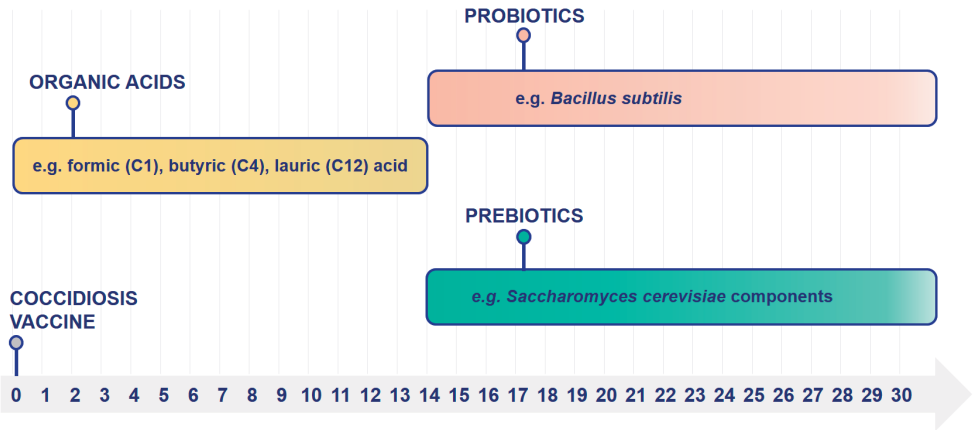

Figure 10. Suggested strategic use of feed additives in commercial broiler flocks, assuming that a live, attenuated anticoccidial vaccine (Paracox-5) is administered on day of hatch and no antibiotic growth promoters or ionophores are added to the feed. Illustrated by S. Granstad (2021).

Organic acid-based feed additives are suggested as performance-enhancing supplements in starter feed. Feed additives containing formic, butyric and lauric acid and/or derivatives thereof provided superior performance results during the first two weeks of rearing.

Probiotic feed additives are possible tools to control proliferation of intestinal *C. perfringens* and support production performance in the period with the greatest expected risk of developing necrotic enteritis. The most efficient probiotics were based on *Bacillus subtilis* strains.

Prebiotic feed additives are proposed as measures to stimulate growth and activity of beneficial microbes present in the intestine, and to increase the success and survival of the probiotics. Products based on components from the yeast *Saccharomyces cerevisiae* were most promising with regard to improving feed conversion.

Based on experiences with Paracox-5 vaccination of day-old broilers in Norway, major intestinal problems are rare during the first two weeks of rearing. Feed additives with organic acids are suggested as tools to enhance growth and feed conversion in this period with low expected infection pressure from *Eimeria* spp. This strategy is based on the assumption that prebiotics and probiotics provide sufficient protection against intestinal disease in the succeeding rearing phase. Ross 308 broilers are slaughtered around 33 days of age in Norway. The use of organic acid-based feed additives in later rearing phases may be of interest in countries where Ross 308 broilers are reared for a

longer period of time or for use in slow-growing breeds, but this use needs to be tested in relevant research models. Further, the predisposing effect of rapid growth on development of necrotic enteritis and cardiovascular and musculoskeletal disorders needs to be considered.

The peak of oocyst shedding in Paracox-5 vaccinated broilers varies substantially between flocks, but is generally expected to occur around the fourth week. Empirical evidence from rearing of Ross 308 broilers in Norway under current production standards indicate that there is a low risk of coccidiosis and/or necrotic enteritis with most clinical cases appearing after three weeks of age (M. Kaldhusdal, Norwegian Veterinary Institute, personal communication, February 17, 2021). It remains unclear whether vaccine strains or wild-type strains of *Eimeria* spp. play a key role in the pathogenesis. Prebiotics and probiotics are suggested as additives in grower feed to prevent negative effects on intestinal health and performance in the period when intestinal distress caused by *Eimeria* spp. and/or *C. perfringens* is most likely to occur. However, the impact of sharp and sudden switches from one type of feed additive to others needs to be evaluated, and it is possible that changes in feed additive administration should be based on a gradual phase-out/phase-in strategy.

The intestinal microbiota of newly hatched chickens is shaped by exposure to microorganisms from the eggshell surface and the hatching environment (Maki et al., 2020; Rinttila & Apajalahti, 2013). It has been suggested that the microbiota starts to develop already prior to hatching, and that both vertical and horizontal transfer of bacteria facilitate microbiota development in embryos (Roto et al., 2016). Introduction of prebiotics early in life is proposed as a strategy to support development of a microbiota that is antagonistic to pathogens, as some pathogens are known to colonise the intestine of young broilers (Ricke et al., 2020). Based on these reports, a potentially successful strategy would be to administer prebiotics from day of hatch in the starter feed of commercial broilers. However, if other feed additives are used simultaneously, possible incompatibilities must be considered. Results from Paper I indicate that organic acids and prebiotics do not necessarily achieve optimum effect when administered together in starter feed. This is exemplified by feed additives based on formic acid/formate (ID 13/ Formi NDF®) and butyric acid/lauric acid/phenolic compound (ID

11/Presan FY®). Used as sole feed additives, both treatments had superior effect on body weight gain and feed conversion ratio during the first two weeks. Both feed additives outperformed narasin in this rearing phase characterized by low intestinal infectious burden. However, when these two feed additives were tested in combination with a *Saccharomyces cerevisiae*-based prebiotic (ID 16/Diamond V XPC®) in treatment ID 24 and 25, the beneficial effect on production performance in the starter feed period disappeared. These results suggest that promising organic acid-based products should be used as sole feed additives to obtain desired effects on performance in rearing phases with low pathogen burden (from 0-14 days as suggested in Figure 10). Future studies are needed to determine whether the beneficial effect of prebiotics and probiotics disappear or diminishes when these feed additives are introduced for the first time in grower feed at a later stage of rearing (from day 14 onwards as suggested in Figure 10). If prebiotics and probiotics must be administered earlier or from day of hatch to obtain desired effects in later rearing stages, their value should be weighed against the value of organic acids. New combinations of other prebiotic compounds and organic acid-based feed additives should be tested in appropriate models to identify possible synergistic combinations.

5.2 Impacts of dietary starch to fat ratio

In Paper II and III, effects of two diets with different inclusion levels of starch and fat were investigated. The ratios of starch to fat were 32:1 and 2.4:1 in the high (HS) and low (LS) diet, respectively. Both treatment groups were challenged with *Eimeria* spp. and consequently exposed to intestinal distress. As indicated by the feed conversion ratio, chickens fed the HS diet were able to utilize the feed more efficiently in the overall study period. Increased understanding of how the diets affected the gastrointestinal environment and why the chickens performed differently could potentially be used to improve feeding strategies in antimicrobial-free broiler production systems.

5.2.1 Starch and fat digestibility

Starch digestibility was higher in the HS group compared with the LS group, irrespective of the higher starch load provided by the HS diet (448 g/kg in HS vs. 231 g/kg in LS). Taking the overall mean digestibility coefficient into consideration, as much as 96% of

the starch in the HS diet was exposed to digestive enzymes, broken down and absorbed by intestinal epithelial cells. This result illustrates the vast capacity of broilers to digest and utilize starch when the starch is accessible to digestive enzymes. The accessibility of starch in the diet depend on factors associated with starch properties. In the HS diet, there was an unintentional higher degree of starch gelatinization compared to the LS diet. Starch gelatinization is a chemical process in which bonds between starch molecules are broken down in the presence of water and heat (Ratnayake & Jackson, 2008). This process allows more water to bind to the starch granule and cause swelling, until the starch granule disintegrates and the amylose (i.e. one of the two components of starch) dissolves into the surroundings. Gelatinization improves the availability of amylose molecules for amylase hydrolysis in the host, potentially leading to higher starch digestibility (Zimonja & Svihus, 2009). A possible explanation for higher starch gelatinization in the HS diet was that the low content of fat gave rise to greater mechanical shear in the pellet press. This resulted in increased friction, higher pellet temperature and subsequently swelling and disintegration of starch granules. In the LS diet, the lubricating effect of rapeseed oil may have caused the reduction in frictional heat, which resulted in a lower degree of starch gelatinization. The observed differences in starch digestibility between the treatment groups in the experiment may thus have been caused by the differences in starch gelatinization.

The use of isolated wheat starch in the HS diet is another factor potentially influencing digestibility. Isolated starch is separated from the grain by a physical process, and appears as a finely ground white powder. This purified form of starch with coherent small particle size is considered to be easily digested by broiler chickens (Rogel et al., 1987), which could also explain the higher starch digestibility of chickens fed the HS diet. Additionally, the starch purification process removes most of the non-starch components that could prevent water infiltration, swelling and disintegration of starch granules (Budny et al., 2005). In this way, the use of isolated wheat starch could have contributed to the higher degree of gelatinization, and consequently the higher digestibility of the HS diet. Isolated wheat starch is relatively costly, and is mainly used as an ingredient in human food (Shevkani et al., 2017). It is used to improve texture, structure and moisture in various foodstuff, but also has a widespread use in non-food productions such as textile, paper, chemical and pharmaceutical industries. The use of

isolated wheat starch as an ingredient in a broiler diet in this study was based on the objective to evaluate effects of starch on various parameters, without other constituents of grains (e.g. non-starch polysaccharides) as potential confounders. However, this starch source is of little practical relevance from a commercial perspective.

Lipids from dietary fat may form inclusion compounds with amylose in the intestine (Huang et al., 2020). In this process, the amylose part of the starch structure is enclosed within lipid structures, which makes the starch less accessible for digestive processes (amylase hydrolysis). The fat content in the LS diet is a reasonable explanation for the lower starch digestibility observed in the treatment group fed this diet. The capacity for fat digestion is not fully developed in young chickens, and factors involved in lipid digestion (e.g. intestinal and pancreatic lipase concentrations, bile salt secretion and fatty acid-binding protein activity) change during the first weeks after hatching (Krogdahl, 1985). Fat digestibility increased with maturation of the intestine (from day 16 to day 29) in both the HS and the LS group. Increased fat digestibility results in fewer lipids available to make complexes with starch. Thus, a gradual age-associated increase in starch digestibility in chickens fed high fat diets would be expected, and this was seen in the LS group.

5.2.2 Intestinal morphology

The surface of the small intestine is covered by projecting structures called villi (Figure 11). These structures are exposed to a wide variety of nutrients, microorganisms and potentially harmful substances and pathogens. A general understanding is that intestinal villi have a 'critical' length, which is dynamic and determined by a balance between the benefit of nutrient absorption and cost of villi maintenance (Moran, 1985). Consequently, villus length is not constant even in healthy birds at a given age. Sampling on three distinct time points in the experiment of Paper III, before, during and after challenge with *Eimeria* vaccine strains, made it possible to study morphological dynamics. The difference in dynamic development of villi length between the two groups implies that the dietary treatments influenced intestinal morphology over time.

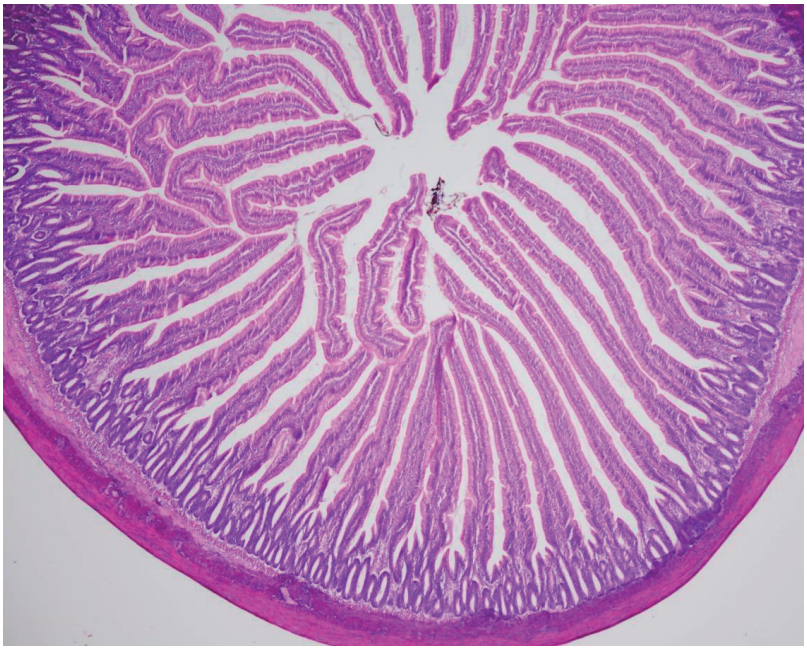

Figure 11. Histological section (40X) stained with haematoxylin and eosin from the transition between duodenum and jejunum in a 16 days old Ross 308 broiler fed a high starch to fat ratio diet. Photo: S. Granstad (2017).

It has been suggested that the capacity to utilize carbohydrates is highly adaptable in fowl (Moran, 1985). Chickens may adjust to changes in dietary starch level by modifications of amylase secretion and intestinal surface area. The small intestinal surface can be increased by elongation of villi. Digestive and structural adaptations associated with a higher dietary starch inclusion level were confirmed in Papers II and III. Amylase activity tended to be higher and small intestinal villi were significantly longer on day 29 in chickens fed the HS diet compared with those fed the LS diet. Increased enzymatic activity and expanded mucosal surface area most likely affected digestion and absorption of starch, and consequently may have contributed to the observed higher starch digestibility and improved feed conversion ratio in this group.

Different *Eimeria* species are site-specific and infect distinct locations of the intestinal tract (Gerhold, 2016). *E. acervulina* is considered one of the most common causes of coccidiosis in broilers, and infects the upper half of the small intestine (mainly duodenum). Shortening of duodenal villi in response to *E. acervulina* infection has been

demonstrated (Assis et al., 2010; Fernando & McCraw, 1973). Also, in a study with a challenge model (inoculation with a ten-fold dose of Paracox-8) with features similar to the challenge used in Paper II and III, the challenged chickens had reduced duodenal villus:crypt ratios compared with negative non-challenged controls in a critical time period during infection (Gholamiandehkordi et al., 2007). Only one of these studies reported dynamic development of villi length after the phase characterized by acute infection. In this study, it was shown that after duodenal villi length had decreased to a minimum on day six post infection, the length of villi gradually returned to normal (Fernando & McCraw, 1973). Moreover, the length of villi in jejunum was less affected by *E. acervulina* infection, but increased rapidly to levels well above controls post infection. It was hypothesised that the noticeable increase in jejunal villi length post infection was a possible compensatory mechanism for loss of effective intestinal surface area in the more heavily parasitized duodenum. A compensatory lengthening of intestinal villi in response to *Eimeria*-challenge was also suggested in Paper III, however, at an earlier time point (days 21-23, i.e. four to six days after *Eimeria*-challenge). The results suggest that the relatively mild challenge with *Eimeria* vaccine strains did not induce enough intestinal damage to cause any serious shortening of villi in the acute phase. On the contrary, the increase in villi length from day 16 to days 21-23 in the LS group propose a temporary lengthening of villi in this group as a response to *Eimeria*-challenge, possibly as an attempt to increase the area of functional mucosal lining. This energy-demanding response may not have been necessary to the same extent in the HS group, which appeared to adapt to the high dietary starch level by a continuous increase of villus length from day 16 to day 29. However, the lack of unchallenged control groups made it impossible to conclude definitively if or to which extent the coccidia had an impact on intestinal morphology in the experiment. Despite this limitation, the results presented in Paper III led to a new hypothesis that longer villi and consequently greater intestinal surface area associated with high-level starch diets make broilers less afflicted by mucosal damage caused by *Eimeria* spp. Future studies should establish whether the manifestation of impairing effects on broiler health and performance is reduced in chickens with increased functional intestinal mucosal lining prior to infection.

5.2.3 *Clostridium perfringens*, necrotic enteritis and short-chain fatty acids

Growth requirements and substrate preferences differ between bacterial species, and the chemical composition of the digesta affects the species distribution of the intestinal microbiota (Apajalahti et al., 2004). As mentioned previously in this thesis, the recognised association between certain cereal grains and necrotic enteritis in broilers may be explained by the ability of *C. perfringens* to degrade starch (Groves & Grounlund, 1969; Shih & Labbe, 1996). Mucosal damage due to coccidiosis or other intestinal homeostasis-disturbing events could result in increased amounts of undigested starch in the digesta. This could create favourable growth conditions for *C. perfringens* and other microorganisms able to utilize starch, and possibly predispose for development of necrotic enteritis. Another suggested explanation for the necrotic enteritis-predisposing effect of cereal grains such as wheat, rye and barley is the relatively high content of water soluble non-starch polysaccharides (NSPs) in such grains, which increases the viscosity of the digesta and enhances the severity of necrotic enteritis in experimental models (Prescott et al., 2016; Shojadoost et al., 2012). In this study, the HS and LS diet contained equal amounts of wheat grains, and the HS diet was supplemented with purified wheat starch instead of grains to increase the starch content. The potentially necrotic enteritis-predisposing effect of NSPs was thus similar in both treatment groups. No difference in *C. perfringens* counts and necrotic enteritis lesions were observed between the HS and LS group. Because the pure wheat starch appeared to be easily digested and effectively removed from the digesta, the ileal starch content of the HS group was only moderately higher than in the LS group, and much lower than in previous studies examining the association between dietary manipulations and ileal starch concentrations (Svihus & Hetland, 2001; Svihus et al., 2010). These findings indicate that the moderate increase in ileal starch level associated with the HS diet did not influence *C. perfringens* proliferation and development of necrotic enteritis in this experiment.

Caecal SCFA concentrations were higher in the HS group six days after the introduction of the experimental diets. The higher microbial fermentation output indicates that the availability of adequate substrates in the posterior intestinal segments was greater in the HS group at an early stage of the experiment. Starch digestibility improved from day six to day 19 after introduction of the experimental diets in the HS group, which likely

reduced the amount of starch available for microbial fermentation and evened the SCFA concentrations in the two dietary treatment groups.

5.2.4 Adaptations to diet

Existing literature and results from Paper II and III demonstrate that broiler chickens can adapt to diets with considerably different nutrient composition. Chickens are known to be able to adjust enzyme activity (amylase, disaccharidases) in response to greater starch levels in the digesta (Kohl et al., 2017; Moran, 1985). This was also implied in Paper II, where the estimated activity of amylase tended to be higher in chickens fed the diet with more starch. Furthermore, chickens are capable of increasing both lipase activity and expression of proteins (intestinal fatty-acid binding proteins) involved in transportation and metabolism of fatty acids in response to higher levels of fat in the diet (Krogdahl, 1985). Higher fat digestibility was observed in the group of chickens fed the diet with greater content of fat in Paper II. Finally, in response to increased dietary starch levels, it has been reported that broilers are able to increase the intestinal absorptive surface area by lengthening of villi (Moran, 1985). The latter characteristic was demonstrated in Paper III, which also produced data indicating that the dietary impact on villus length was modified by bird age, time elapsed from introduction of experimental diets and/or *Eimeria*-challenge.

6. Main conclusions

The study of commercially available non-antibiotic feed additives in Paper I demonstrated that:

- The group of probiotic feed additives improved overall production performance, and improved production performance and reduced *C. perfringens* counts in a time interval comprising infection with coccidia. The probiotics group impaired weight gain during the first two weeks post-hatch.
    - The best-performing probiotic product in terms of production results was based on a *Bacillus subtilis* strain (Gallipro®).
    - The best-performing probiotic product with regard to *C. perfringens* reduction was based on the *Bacillus subtilis* PB6 strain (Clostat®).
- The group of prebiotic feed additives improved overall production performance and production performance in a time interval with *Eimeria*-challenge. All four tested prebiotic products were based on components from the yeast *Saccharomyces cerevisiae*.
    - The best-performing prebiotic products in terms of production results were based on yeast cell wall extracts containing (among other components) β-1,3/1,6 glucans (Agrimos® and MacroGard®).
    - The only prebiotic product with a reducing impact on *C. perfringens* was based on dehydrated *Saccharomyces cerevisiae* culture with whole cells, metabolites and medium nutrients (Diamond V XPC®).
- The group of phytogenic feed additives improved overall feed conversion ratio and reduced *C. perfringens* counts in a time interval with *Eimeria*-challenge.
    - The only phytogenic product with a combined overall improvement of feed conversion and reduced *C. perfringens* counts was based on oleoresins from turmeric and chilli peppers (Xtract Nature®).
    - The only phytogenic feed additive that improved production performance in a time interval with *Eimeria*-challenge was based on essential oils (Digestarom PEP 150 MGE®).
- The group of organic acid-based feed additives improved production performance during the first two weeks post-hatch, improved overall production performance, and increased weight-gain in a time interval comprising infection with coccidia.

    - The best-performing organic acid product with regard to production results was based on diformate derived from Na-formate and formic acid (Formi NDF®).
- Among the mixed feed additives, three feed additives based on combinations of organic acids and phytogenic components showed beneficial effects:
    - A product based on benzoic acid and essential oils improved production performance in a time interval with *Eimeria*-challenge (Crina Poultry Plus®).
    - Short- and medium-chain fatty acids (including C4 and C12) in combination with (among other components) a phenolic compound improved production performance during the first two weeks post-hatch, improved overall production performance, and increased weight-gain in a time interval comprising infection with coccidia (Presan FY®).
    - The only feed additive with organic acids (short- and medium-chain fatty acids including monoglycerides of propionic, butyric, caprylic and capric acid) that reduced *C. perfringens* counts also contained essential plant oils (FRA Gut Balance®). This product did not affect production performance significantly.
- A reduction in *C. perfringens* counts and a beneficial effect on feed conversion ratio did not always correlate. Effects on the host and on the intestinal microbiota are suggested as other possible mechanisms of action of non-antibiotic feed additives that may influence production performance.
- Both synergistic effects (i.e. the combined effect was greater than the effects achieved when administered separately) and antagonistic effects (i.e. the combined effect was less than the effects achieved when administered separately) were observed with the tested combinations of specific feed additive products.

The results obtained from the study of dietary starch to fat ratios in *Eimeria*-challenged broilers in Paper II and III showed that:

- The hypothesis that a high starch to fat ratio impairs nutrient digestibility, production performance and gut health was not verified.
    - Higher dietary starch inclusion level augmented starch digestibility.
    - Chickens fed the high starch to fat ratio diet utilized the feed more efficiently (measured by feed conversion ratio).

- Differences in dietary starch to fat ratio did not have an impact on *C. perfringens* counts, toxin profile or necrotic enteritis prevalence.

- The rejection of the hypothesis needs to be evaluated in light of two possibly confounding factors:
  - A higher degree of starch gelatinization in the high starch to fat ratio diet.
  - Use of isolated wheat starch in the high starch to fat ratio diet.
- Microbial fermentation output measured as total caecal SCFA level was greater in chickens fed the diet with higher dietary starch level prior to *Eimeria*-challenge, but this difference between the diets was not present during peak excretion of oocysts.
- Higher dietary starch level was associated with increased length of small intestinal villi on day 19 after the introduction of experimental diets. This association was not present during peak excretion of oocysts on days 11-13 after the introduction of experimental diets. These findings suggest that the broilers responded with structural intestinal adaptations to dietary starch level, a response that appeared to be modified by *Eimeria*-challenge.

7. Future perspectives

Results from Paper I can be used to select promising active components and non-antibiotic feed additive combinations in future studies on alternatives to in-feed antimicrobials with antibiotic properties. Mechanisms of action are unknown for several of the active components in these feed additives, and more knowledge of functional principles could increase their value in broiler rearing without ionophores and AGPs. Field trials would be useful to establish evidence-based advice on the strategic use of feed additives in commercial, antimicrobial-free broiler flocks.

Results from Paper II and III indicate that there might be benefits of high levels of starch in broiler diets. To allow for high starch inclusion levels in diets without starch digestibility being a limiting factor, evaluations of practically relevant starch sources and feed processing conditions are required. Broiler diets with larger amounts of cereal grains with climatic production potential in Norway (e.g. barley and oats) could contribute to a more sustainable Norwegian broiler production. Studies of alternative dietary starch sources such as barley and oats, where efforts are made to increase the starch digestibility, would be useful to clarify the potential of increased dietary starch levels in broilers reared without in-feed antimicrobials. Factors known to influence starch digestibility (i.e. starch source, level of grinding and degree of gelatinization as emphasised in Paper II and III) are also known to affect other chemical and physical properties of the feed, and the benefit of increased starch digestibility might be outweighed by negative side-effects on intestinal health.

Global economic losses caused by coccidiosis and necrotic enteritis in broiler production are estimated to be as much as ~ US$ 14 and 6 billion each year, respectively (Blake et al., 2020; Wade & Keyburn, 2015). These estimates are calculated with a high degree of uncertainty, and the predicted losses may overlap due to the association between disease caused by *Eimeria* spp. and *C. perfringens*. Nevertheless, coccidiosis and necrotic enteritis are considered to be key production-limiting diseases in broiler production worldwide. It has been argued that ionophores are necessary to manage coccidiosis, and some have raised concerns about more frequent use of antibiotics for treatment of necrotic enteritis if the prophylactic use of ionophores was restricted or banned

(Kadykalo et al., 2018; Karavolias et al., 2018). This concern was also relevant in the phasing-out period of ionophores as feed additives in Norway. However, annual reports of antimicrobial consumption revealed that the use of antibiotics in broiler production continued to be low in the years following the abolition of narasin, and less than 0.1% of Norwegian broiler flocks were subjected to treatment with therapeutic antibiotics in 2019 (NORM-VET, 2020). Vaccination of day-old chicks against coccidiosis has been implemented as a prophylactic measure (Nortura SA, 2017). Furthermore, narasin has in some cases been used to treat necrotic enteritis as an alternative to therapeutic antibiotics, administered for a period of 3-5 days with the same dosage as when used prophylactically (A. Løvland, Nortura SA, personal communication, March 1, 2021). Optimized feed composition, high quality standard of management practices and anticoccidial vaccination are likely factors that so far have contributed to improve the sustainability of broiler production without prophylactic use of ionophores in Norway. Experiences from the abolition of ionophores as feed additives in the Norwegian broiler industry may be valuable to other countries with comparable production systems. Furthermore, the possibility of using ionophores therapeutically to control necrotic enteritis outbreaks should be explored in more countries, considering that the prophylactic use of ionophores in recommended doses may have a negative impact on broiler performance under certain circumstances (e.g. in the absence of coccidiosis and during warmer months of the year) (Chapman et al., 2010; Reece, 1988). In a global perspective, improved control of coccidiosis and necrotic enteritis is likely to be a prerequisite for a sustainable broiler production without prophylactic use of ionophores and with minimal use of therapeutic antibiotics. Effective, non-antibiotic disease-preventive measures associated with feed composition, non-antibiotic feed additives, management practices, biosecurity and vaccination are required.

Antimicrobial resistance is a serious global threat to public health, and if the current trend continues, we may approach a future where there are few antibiotics left to treat bacterial infections. The extensive use of antimicrobials in the global livestock sector is of considerable concern (Humphreys & Fleck, 2016). In some countries, livestock industries have evolved rapidly into highly intensified production systems, requiring antimicrobials to maintain health and productivity. Antimicrobial use in food animals in a group of these transitioning countries (BRICS, i.e. Brazil, Russia, India, China, South

Africa) is expected to double in the period 2010 to 2030 (Van Boeckel et al., 2015). The use of medically important antibiotics is the primary concern in several countries, and efforts to reduce the use of these substances are of utmost importance. However, it should not be an excuse to neglect potentially negative implications of using ionophores as feed additives on a routine basis. Future research should aim to establish the specific contributions of ionophores in development, spread and persistence of AMR.

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9. Enclosed papers I – III

Paper I

animals

MDPI

Article

Effect of Feed Additives as Alternatives to In-feed Antimicrobials on Production Performance and Intestinal *Clostridium perfringens* Counts in Broiler Chickens

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Simple Summary: For many years, antibiotics were added to chicken feed to prevent disease and promote growth. This practice has been banned or voluntarily abolished in many countries. However, most countries still allow the use of in-feed ionophorous coccidiostats, which are drugs that possess both antiparasitic and antibacterial properties. Concerns related to antimicrobial resistance have led to increased focus on broiler chickens raised without the use of any antimicrobial agents, and the interest in non-antibiotic feed additives with beneficial effects on gastrointestinal health and productivity is growing. In this study, feed additives with active components belonging to the product classes probiotics, prebiotics, phytogenics and/or organic acids were assessed for their effect on intestinal health and production performance in broiler chickens. Collectively, the group of non-antibiotic feed additives improved gut health and performance, but not to the same extent as the ionophorous coccidiostat narasin. Probiotics and prebiotics had the overall best performances during coccidia challenge, phytogenics improved overall feed conversion and reduced counts of the intestinal bacterium *Clostridium perfringens*, and organic acids increased weight gain independent of age. This study provides comparable and unbiased results from testing of alternatives to antibiotics in a uniform experimental model highly relevant to commercial conditions.

Abstract: Numerous non-antibiotic feed additives (alternatives to antibiotics, ATAs) have been marketed, but few have been evaluated under uniform testing conditions modelling commercial flocks. We compared 24 ATA treatments and the ionophorous coccidiostat narasin against a diet without any feed additives. Feed conversion ratio and body weight gain were registered from day 0 to 28 in Ross 308 chickens housed on litter floor. The chickens were challenged with *Eimeria* spp., and cecal *Clostridium perfringens* (CP) counts were investigated. Active components from all ATA classes had a positive impact on intestinal health or production performance. Whereas narasin had a strong CP-reducing effect in combination with performance-promoting impact, only two ATA treatments achieved significantly beneficial effects on CP counts as well as feed conversion during the time span following *Eimeria* challenge. Active components present in these two treatments include a *Bacillus subtilis* probiotic strain, short- and medium-chain fatty acids and *Saccharomyces cerevisiae* components. Different ATA classes had beneficial impact during distinct rearing phases and on

specific performance targets, suggesting that optimizing combinations and use of active components can make ATAs even more useful tools in broiler rearing without the use of in-feed antimicrobials. Further studies of promising ATAs and ATA combinations are required.

Keywords: broilers; feed additives; probiotics; prebiotics; phytogenics; organic acids; anticoccidials; necrotic enteritis; *Clostridium perfringens*; production performance

1. Introduction

The use of antimicrobial growth promoters (AGPs) was abolished in Sweden, Norway and Denmark in 1986, 1995 and 1998–1999, respectively [1]. As a response to this development, the use of ionophorous coccidiostats (e.g., narasin) in broiler feeds increased and became more important than before [2]. In 2006, the European Union implemented a total ban of AGPs, meaning that antimicrobials other than coccidiostats and histomonostats were no longer allowed as feed additives in the poultry industry [3,4]. Coccidiostats like narasin and other ionophores are still approved in the European Union for control of coccidiosis caused by the parasitic protozoans *Eimeria* spp. in poultry.

Ionophores are primarily approved for control of coccidiosis but may also have antibacterial and antiviral properties [5]. Narasin has a well-known inhibitory effect on the potential pathogen *Clostridium perfringens* (CP), which is associated with the intestinal disease necrotic enteritis (NE) in broiler chickens [6,7]. Selected ionophores have been suggested as novel antimicrobial agents to control infectious diseases in animals as alternatives to antimicrobial classes used to treat human disease [8]. Furthermore, concerns have been raised regarding the possibility that the use of narasin and other ionophores could be associated with bacterial resistance against antimicrobials used in human medicine, and that resistant bacteria could spread to humans both by direct contact with animals and through food supply [2,9]. These considerations have led to increased focus on conventional broilers raised without the use of any in-feed antimicrobial agents, including AGPs as well as ionophores and other coccidiostats. In 2015/2016, the Norwegian broiler industry abolished the routine use of in-feed coccidiostats, including narasin [10].

The former widespread practice of supplementing broiler feeds with AGPs was mainly based on the favorable influence of these compounds on production performance [2]. Impaired production performance leading to increased production costs is a main concern associated with rearing broilers without in-feed antimicrobials. The traditionally most commonly used AGPs are predominantly active against gram-positive bacteria [11], and many of these antimicrobials have been shown to suppress the proliferation of CP in vivo [12,13] and in vitro [14–16]. Several studies report an association between increased numbers of intestinal CP and growth depression in chickens [12,17,18], and collectively these findings suggest that antibacterial activity against CP may be involved in the 'antibiotic growth effect'. Development of NE and a subclinical form of this disease is associated with impaired production performance, cholangiohepatitis and high numbers of intestinal and fecal CP [19–21]. Infection with *Eimeria* spp. is considered an important predisposing factor for CP proliferation and development of NE in chickens [22,23].

The interest in non-antibiotic feed additives (hereafter: alternatives to antibiotics, ATAs) that might facilitate the abolishment of continuous use of in-feed AGPs and coccidiostats has increased during the recent years. Numerous new feed additives have reached the global poultry feed market. Different ATAs, including products based on probiotics, prebiotics, phytogenics and/or organic acids, claim to exert beneficial effects related to productivity, intestinal functions and intestinal health in broiler chickens.

Probiotics are based on non-pathogenic and non-toxigenic live microorganisms (e.g., bacteria or yeasts) supposed to provide health benefits to the host. Possible modes of action of probiotics include colonization of the intestine, competitive exclusion of other microorganisms, production of

specific metabolites and stimulation of the immune system [24]. Two categories of probiotics are non-spore forming bacteria (e.g., *Lactobacillus* spp., *Enterococcus* spp., *Bifidobacterium* spp.) and bacterial spore formers (e.g., *Bacillus* spp.) [25]. Regulatory agencies have been reluctant to approve undefined microbial products due to the uncertainty of a consistent composition of the products. This concern has paved the way for defined probiotic products based on one or a few known strains.

Prebiotics are non-digestible feed ingredients assumed to stimulate proliferation and/or activity of intestinal microorganisms, which leads to beneficial physiological responses in the host [26]. Intake of prebiotics may increase the number of specific microbes and change the composition of the intestinal microbiota [27]. Examples of prebiotic compounds are complex carbohydrates derived from plants or yeasts, such as fructooligosaccharides (FOS), mannanoligosaccharides (MOS) and β-glucans [28,29]. In addition to selective promotion of beneficial bacteria, suggested modes of action of prebiotics are blocking pathogen adhesion, altering gene expression, affecting gut morphological structure and immunomodulation [29].

Phytogenic feed additives are based on bioactive compounds derived from plants, and a multitude of such plant products can broadly be classified as herbs or spices [28]. Examples of biologically active components and substances from plants are essential oils, oleoresins, tannins, saponins, flavonoids, alkaloids and resin acids. Various functions among plant-based products have been suggested, including antimicrobial, antiviral, antioxidative, anti-inflammatory and flavoring effects [30]. The compositional variation is considerable due to biological factors such as plant species, growing conditions, climate, harvest and manufacturing processes, and it is thus challenging to identify and evaluate the functional basis of this broad group of active components [31].

Organic acids of various lengths and their corresponding salts or esters are widely used as feed additives in livestock production and can be used individually or as blends of multiple acids. They may vary considerably in functionality due to number of carbon atoms and may be aliphatic or aromatic. Many organic acids consist of carboxylic acids and are natural constituents of animal or plant tissue or products of microbial fermentation. Industrially produced organic acids often come as salts or esters and in a coated or encapsulated form [31]. Carboxylic acids with an aliphatic chain are designated fatty acids. The subgroup short-chain fatty acids (SCFAs, 1–5 carbon atoms; C1–C5) are aliphatic compounds produced in nature by microbial fermentation of carbohydrates in the hindgut of humans and animals. The subgroup medium-chain fatty acids (MCFAs, 6–12 carbon atoms; C6–C12) are aliphatic compounds formed in nature predominantly in plants and extra-intestinal animal tissues. Suggested effects of organic acids are antibacterial activity through pH-regulation and changes in microbiota composition, immunomodulatory action and stimulation of the gut mucosa [28,29,31]. The heterogeneity of this feed additive category makes it difficult to define common properties and function, and the effects of different organic acids may vary considerably. It has been proposed that SCFAs can act directly upon the cell wall of gram-negative bacteria, and that fatty acids with longer chains can incorporate themselves into the cell membrane of gram-positive bacteria and promote leakage [32].

A multitude of studies on the impact of alternative feed additives in broiler chickens have been published. However, most studies focus on only one or a few additives within one or two ATA classes. Furthermore, these studies often differ with regard to a number of factors that may influence the results (e.g., housing of chickens, number of replicates and challenge), which makes it difficult or impossible to compare results across studies. Another problematic issue is publication bias that occurs when only results that show significant findings are reported [33]. These considerations make it relevant to study the effect of ATAs under uniform testing conditions.

The present study was conducted in order to examine the effect of commercially available ATAs from four different product classes on production performance and cecal CP counts. Feed additives were selected on the basis of being marketed with claimed beneficial effects on production performance, intestinal function and/or intestinal health in poultry. Production performance was recorded during two separate age levels; days 0–14 and days 14–28. CP counts were recorded during the fourth week of rearing, four to six days after challenge with *Eimeria* spp.

The aims of the study were to (a) evaluate the performance of the collective ATA group, (b) compare effects of classes of ATAs (probiotics/prebiotics/phytogenics/organic acids) and (c) identify active components or component combinations with beneficial effects on production performance and CP counts, with emphasis on the time span following *Eimeria* challenge.

2. Materials and Methods

2.1. Animals and Housing

Six trials were carried out at Scandinavian Poultry Research in Våler, Hedmark, Norway, using one-day-old Ross 308 broiler chickens obtained from a commercial hatchery (Nortura Samvirkekylling, Våler, Norway). The chickens were housed in floor pens of 5.6 m^2 on new wood shavings in a climate-controlled poultry research facility, with a 50/50 female-to-male ratio per pen. Water and pelleted feed were given *ad libitum*. The chickens were exposed to light for 23 h a day on the first two days. For the rest of the experimental period, the chickens were exposed to light during 2 × 8 h a day, interrupted by 4 h periods of darkness. Apart from a 10-fold dose of Paracox-5 vet. on day 17 or 18, no vaccines were administered throughout the study. The study period lasted from day of hatch until day 28. Animal experiments were approved by the national animal research authority (Norwegian Food Safety Authority, approval ID 8179), and performed in accordance with national and international guidelines for the care and use of experimental animals.

2.2. Experimental Design

In each of the six trials, a total of 5280 one-day-old Ross 308 broiler chickens were randomly allocated into six experimental groups, each group comprising 11 replicate pens with 80 chickens per pen. All trials had similar design, and included four treatment groups receiving feed with a specific ATA product or a combination of two ATA products, a positive control group (NAR) receiving feed with the polyether ionophore and coccidiostat narasin (Monteban, Elanco Animal Health, Greenfield, IN, USA), and a negative control group (NEG) receiving feed with neither antimicrobial feed additives (AGPs or coccidiostats) nor ATA products. Feed additives were added to the feeds at an inclusion rate recommended by the manufacturers. No AGP products were included in this study, and narasin was used as a sole coccidiostat in the NAR group. The chickens were fed wheat-based starter and grower diets based on Ross Broiler Nutrition Specifications adapted to Norwegian broiler production from 0 to 14 and 14 to 28 days of age, respectively (Table 1).

In the five initial trials, 20 commercially available ATA products were evaluated individually for their effect on production performance and cecal CP counts. In the sixth trial, combinations of two ATA products per treatment group were evaluated using the same outcome variables. Products included in the sixth experiment were selected for testing due to promising impact on either production performance or CP counts in the five initial experiments. Products with positive effects on production performance were combined with products with CP reducing effect in order to study potential synergy effects. Descriptions of active ingredients and dose levels of the feed additives and feed additive combinations tested are listed in Table 2. Composition of the products and dosage levels are based on information given by the feed additive manufacturers on their web sites or as a response to our request.

On day 17 (one trial) or 18 (five trials) post hatch, all treatment groups in all six trials were challenged with a 10-fold dose of Paracox-5 vet. (MSD Animal Health, Boxmeer, the Netherlands) containing live, sporulated oocysts from five attenuated strains of *Eimeria* spp. (one precocious line each of *Eimeria acervulina* [approximately 5750 oocysts per broiler], *Eimeria mitis* [approximately 11,500 oocysts], and *Eimeria tenella* [approximately 5750 oocysts], and two precocious lines of *Eimeria maxima* [approximately 3450 oocysts]) in the drinking water.

Table 1. Diet composition [1].

Chemical Composition	Starter Diet [2]	Grower Diet [3]
(g/kg feed)	0–14 days	14–28 days
Dry matter	887.2	881.3
Crude protein	239.6	222.0
Crude fat	67.8	99.6
Crude fiber	30.3	29.0
Nitrogen-free extracts	493.7	479.0
Ash	55.8	51.7
Lysine	14.0	12.9
Methionine + Cysteine	11.6	11.1
Threonine	9.4	9.0
Tryptophan	2.7	2.5
Arginine	13.8	12.7
Calcium (Ca)	9.2	7.4
Phosphorus (P)	6.3	5.9
Sodium (Na)	1.4	1.6
Potassium (K)	7.7	7.4
Chloride (Cl)	2.3	2.2
Magnesium (Mg)	1.6	1.6
NSP enzymes [4] and phytase	0.15	0.15
Metabolizable energy (MJ/kg)	12.13	12.78

[1] Mean values from diets in six trials. [2] Vitamins and minerals: Cu 15 mg/kg; Zn 82 mg/kg; Mn 126 mg/kg; Se 0.27 mg/kg; I 1.04 mg/kg; Fe 52 mg/kg; Vit.A 9575 IU; Vit.E 96 IU; Vit.D3 4994 IU; Vit.K 7.0 mg/kg; Vit.B1 4.2 mg/kg; Vit.B2 7.3 mg/kg; Vit.B3 59.7 mg/kg; Vit.B5 20.0 mg/kg; Vit.B6 12.0 mg/kg; Vit.B12 0.02 mg/kg; biotin 2.1 mg/kg; folic acid 2.9 mg/kg; choline chloride 1726 mg/kg. [3] Vitamins and minerals: Cu 15 mg/kg; Zn 82 mg/kg; Mn 128 mg/kg; Se 0.27 mg/kg; I 1.05 mg/kg; Fe 53 mg/kg; Vit.A 9488 IU; Vit.E 81 IU; Vit.D3 4983 IU; Vit.K 5.6 mg/kg; Vit.B1 3.6 mg/kg; Vit.B2 6.8 mg/kg; Vit.B3 54.0 mg/kg; Vit.B5 18.0 mg/kg; Vit.B6 11.0 mg/kg; Vit.B12 0.02 mg/kg; biotin 2.4 mg/kg; folic acid 2.7 mg/kg; choline chloride 1500 mg/kg. [4] Non-starch polysaccharide enzymes.

Table 2. Treatment ID, class of feed additives, active components and inclusion rate of feed additive products.

ID [1]	Class [2]	Active Components and Product Description [3]	Dosage [4] (Starter/Grower)
0	NEG	None	–
1	NAR	Narasin (100 g narasin/kg additive)	700/700
2	PRO	*Lactobacillus farciminis* CNMA 67/4R strain (1×10^9 cfu/gram additive)	500/500
3	PRO	*Bacillus subtilis* PB6 strain (2×10^8 cfu/gram additive)	500/500
4	PRO	One *Bacillus subtilis* strain, material no. 671265 (1.6×10^9 cfu/gram additive)	500/500
5	PRO/PRE	*Enterococcus faecium* DSM 16211 (jejunum isolate), *Bifidobacterium animalis* DSM 16284 (ileum isolate), *Lactobacillus salivarus* DSM 16351 (caeca isolate) with mix ratio 3:1:6 (total cfu/gram: 2×10^8), plant-derived fructooligosaccharides from inulin	1000/1000
6	PRE	*Saccharomyces cerevisiae* cell wall extracts (including typ. 25% β-1,3/1,6 glucans and min. 24% mannanoligosaccharides)	1000/1000
7	PRE	*Saccharomyces cerevisiae* cell wall extracts (including min. 60% purified β-1,3/1,6 glucans)	250/250
8	OA/PFA	Benzoic acid (80%–83%) and a blend of essential oils (including thymol 1.0%–1.9%, eugenol 0.5%–1.0%, and piperine 0.05%–0.1%)	300/300
9	PFA	Essential oil blend (min. 31.9%, including carvacrol, thymol, anethol and limonene)	150/150
10	OA	Medium-chain fatty acids (C6, C8 and C10)	1600/1600

Table 2. *Cont.*

ID [1]	Class [2]	Active Components and Product Description [3]	Dosage [4] (Starter/Grower)
11	OA/PFA	Short- and medium chain fatty acids (including C4 and C12), phenolic compound and organic acids	1500/1500
12	OA	Tri- and diglycerides of butyric acid (C4)	1000/1000
13	OA	Diformate derived from C1 (57% Na-formate, 39% formic acid)	3000/3000
14	OA	Lactylates (C12 and C14 esterified with lactic acid)	750/750
15	OA/PFA	Short- and medium-chain fatty acids (including monoglycerides of C3, C4, C8 and C10) and essential oils (mainly cinnamon aldehyde)	3000/2500
16	PRE	Dehydrated *Saccharomyces cerevisiae* culture with whole cells, metabolites and medium nutrients	1250/620
17	OA/PFA	Glycerol-esterified short- and medium-chain fatty acids (including C3, C4, C8 and C10) and 6% phytogenics (including essential oils, saponins and bitter and pungent substances)	750/750
18	PFA	Phytogenics including alkaloids, saponins, thymol and glyco-components derived from Yucca plants	2000/1000
19	PRE	*Saccharomyces cerevisiae* cell wall extracts (primarily mannanoligosaccharides)	800/400
20	PFA	Tall oil fatty acids from coniferous trees, including resin acids (8%–9%)	1000/1000
21	PFA	Oleoresins from turmeric (*Curcuma longa*) (4.4%) and chili peppers (genus Capsicum) (4.4%)	100/100
22	PRO/PRE +PRE	Active components of ID 5 and ID 7	1000/1000 250/250
23	PRE +PFA	Active components of ID 7 and ID 21	250/250 100/100
24	OA/PFA +PRE	Active components of ID 11 and ID 16	1500/1000 1250/625
25	OA +PRE	Active components of ID 13 and ID 16	3000/2000 1250/625

[1] Treatment ID number. [2] NEG = negative control, NAR = positive control, PRO = probiotics, PRE = prebiotics, PFA = phytogenics, and OA = organic acids. [3] Based on available information from the product manufacturers. [4] Amount added product given as grams/ton feed in starter and grower diets.

2.3. *Clostridium Perfringens* Quantification

On days 4, 5 and 6 after *Eimeria* challenge, 11 chickens per treatment group (1 chicken from each replicate pen) were randomly selected and humanely euthanized by cranial stunning immediately followed by cervical dislocation before necropsy. Samples of cecal contents were collected in sterile stomacher bags and directly subjected to cultivation in order to quantify CP. In brief, the samples were diluted 1:100 in peptone saline water (0.1% peptone, Difco Laboratories Inc., Detroit, US and 0.85% NaCl) and homogenized for 30 s in a stomacher (Bagmixer 400 CC, Interscience, Saint Nom, France). Serial dilutions were made with non-buffered peptone water until a dilution of 10^{-6} was reached. Aliquots of 100 µL from the dilutions 10^{-2}, 10^{-4} and 10^{-6} were plated onto sheep blood agar plates (Oxoid Blood Agar Base No.2 and 5% sheep blood, manufactured by the Norwegian Veterinary Institute, Oslo, Norway). The plates were incubated anaerobically at 37 °C for 24 h (Genbox anaer, Biomérieux, Marcy-l'Étoile, France). Single colonies with double hemolysis were counted, and colony-forming units per gram (cfu/g) cecal contents were calculated based on the given dilution. Typical colonies were selected for pure cultivation and later confirmed as CP by a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Bruker Corp., Billerica, MA, USA).

2.4. Post Mortem Examination

The small intestine of all chickens that were sampled for CP quantification was opened longitudinally and examined for pathological changes indicating NE, and scored as follows (modified from [34]): necrotic enteritis negative with no macroscopic mucosal ulcers or pseudomembranes, or necrotic enteritis positive with minimum one mucosal ulcer or pseudomembrane.

2.5. Production Performance Measurements

The amount of feed per pen was weighed when allocated and remaining feed was weighed before being discarded at feed change and at the end of the experiment. Accumulated feed intake per pen from days 0 to 14, 14 to 28 and 0 to 28 was calculated. Total live chicken weights per pen were recorded on days 0, 14 and 28, and mean body weight gain (BWG, g/chicken) and mean feed conversion ratio (FCR, g feed intake/g weight gain) per pen were calculated.

2.6. Statistical Analysis

Data on production performance and CP counts were examined on three different levels; (a) the impact of ATAs as one collective group (group level), (b) the impact of classes of ATAs (class level), and (c) the impact of individual ATA treatments (treatment level). On all levels, ATAs and the positive control with narasin-supplemented feed (NAR) were compared against the negative control with no feed additive (NEG). Frequencies of broilers with NE lesions were analyzed only on group level using Pearson's chi-squared test in Stata version 14.2 (StataCorp LLC, College Station, TX, USA). Production performance and CP count data were analyzed using regression analyses in R version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria).

Production performance data were analyzed with pen as the unit of concern. Body weight gain and feed conversion ratio was obtained in the periods 0–14 days, 14–28 days and 0–28 days for groups, classes and treatments tested in six trials. The outcome from the six different trials could not be compared directly due to intertrial variability. In order to validly compare results from six different trials, it was necessary to control for the effect of trial in the statistical analysis. The principle approach to achieve such control was to use the results from NEG in each of the six trials as indicators of trial effect. A mixed-effects model (1) with only intercept (a) was used to obtain a trial-specific random effect (ε_{Trial}) for each outcome variable (y_{Neg}) per trial based on results from NEG using the package *lme4* in R [35].

$$y_{Neg} = a + \varepsilon_{Trial} \quad (1)$$

For each of the outcome variables (y), results achieved in the different trials were adjusted with a value equal to the random effect obtained for the respective trial. Results across trials were compared using regression analysis (2) with ATA group/class/treatment (x) as fixed-effect variable and trial-specific random-effects from NEG as offset variable (ε_{Trial}). b represents the estimated parameters in the model.

$$y = \varepsilon_{Trial} + b \cdot x \quad (2)$$

The necessity of adjustment for trial effect was calculated by the intraclass correlation coefficient (ICC), which is variance explained by the random effect divided by total variance of the residuals for the model based on all observations from NEG. Extreme outlier pens that were highly influential on the estimated regression results were identified using the function *outlierTest* from the package *car* in R [36]. Residuals from the regression models were visually inspected using the functions *qqnorm* and *qqline* in R and found to follow a normal distribution. The production performance results were reported in tables as means with standard deviation. Differences from NEG with $p < 0.05$ were accepted as statistically significant differences.

CP counts in cecal samples were analyzed with individual chicken samples as unit of concern. Since the residuals from the regression model did not follow a normal distribution, the CP count

numbers were log transformed in order to fulfil this requirement. The effect of trial was controlled by adjusting for obtained random effect as described above, and subsequently regression analysis with ATA group/class/treatment as fixed-effect variable and trial-specific random-effects from NEG as offset variable was conducted. The results were reported in tables as mean $\log_{10}$ colony forming units per gram cecal content. Estimated mean $\log_{10}$ CP counts with 95% confidence interval for each treatment were presented in a graph where feed additive classes are indicated with different colors.

3. Results

3.1. Impact of the Collective ATA Group on Necrotic Enteritis, Intestinal CP Counts and Production Performance

Broilers with necrotic enteritis lesions during days 4–6 after *Eimeria* challenge constituted 8.1% among chickens from the NEG group (no feed additive, n = 198 chickens), 4.4% in the collective ATA group (24 ATA treatments, n = 792 chickens) and 0.5% in the NAR group (in-feed narasin, n = 198 chickens). Statistical analyses indicated significant difference in NE occurrence between the NEG group and the ATA group ($p < 0.05$), and between the ATA group and the NAR group ($p < 0.01$).

The ATA group reduced CP counts in intestinal contents from $\log_{10}$ 6.09 to $\log_{10}$ 5.63 cfu/g ($p = 0.005$), corresponding to a 65% reduction in non-transformed counts (Table 3). This substantial reduction was, however, moderate as compared to the very strong effect of narasin (from $\log_{10}$ 6.09 to $\log_{10}$ 2.92 cfu/g ($p < 0.001$), corresponding to a 99.9% reduction in non-transformed counts).

Table 3. Body weight gain, feed conversion ratio and *Clostridium perfringens* counts for negative control, narasin and alternatives to antibiotics [1].

Group	Days 0–14		Days 14–28		Days 0–28		CP Counts $\log_{10}$ cfu/g
	BWG g	FCR g/g	BWG g	FCR g/g	BWG g	FCR g/g	
NEG [2]	474 ± 4	1.098 ± 0.006	1240 ± 9	1.338 ± 0.005	1714 ± 11	1.248 ± 0.003	6.09 ± 0.14
NAR [3]	488 ± 6 $p = 0.032$	1.064 ± 0.008 $p < 0.001$	1337 ± 12 $p < 0.001$	1.273 ± 0.007 $p < 0.001$	1825 ± 16 $p < 0.001$	1.192 ± 0.005 $p < 0.001$	2.92 ± 0.20 $p < 0.001$
ATAs [4]	478 ± 5 $p = 0.419$	1.087 ± 0.006 $p = 0.079$	1275 ± 10 $p < 0.001$	1.317 ± 0.006 $p < 0.001$	1753 ± 12 $p = 0.002$	1.232 ± 0.004 $p < 0.001$	5.63 ± 0.16 $p = 0.005$
ICC [5]	0.35	0.61	0.43	0.35	0.42	0.28	0.08

[1] Results are reported as means ± standard deviation. Body weight gain (BWG) in grams/chicken, feed conversion ratio (FCR) in grams feed intake/grams weight gain and *Clostridium perfringens* (CP) counts as $\log_{10}$ colony forming units/gram cecal content. [2] Negative control (no feed additive); production performance data based on n = 66 pens, and CP data based on n = 198 individual chicken samples. [3] Narasin; production performance data based on n = 66 pens, and CP data based on n = 198 individual chicken samples. [4] Alternatives to antibiotics treatments; production performance data based on n = 264 pens, and CP data based on n = 792 individual chicken samples. [5] Intraclass correlation coefficient.

Both the ATA group and the NAR group had strongest beneficial impact on production performance during days 14–28, i.e., the age interval characterized by intestinal stress induced by *Eimeria* challenge on day 17 or 18. The collective ATA group demonstrated a 1.6% improvement ($p < 0.001$) in FCR during days 14 to 28 ($FCR_{14–28}$) and a 2.8% increase ($p < 0.001$) in BWG during days 14 to 28 ($BWG_{14–28}$) compared to the NEG group (Table 3). The beneficial effect of the ATA group on production performance was not as pronounced as the positive effect of narasin (4.9% improved $FCR_{14–28}$ and 7.8% increased $BWG_{14–28}$).

3.2. Impact of ATA Classes on Intestinal CP Counts and Production Performance

Four ATA classes (probiotics, PRO; prebiotics, PRE; phytogenics, PFA; organic acids, OA), a set of treatments each based on more than one ATA class (mixed products, MIX) and NAR (i.e., narasin) were compared with NEG (i.e., no feed additive) (Table 4). Although all ATA classes demonstrated

a reducing effect on numbers of CP per gram intestinal contents, only two classes (PFA and PRO) showed statistically significant reduction ($p < 0.05$). The estimated reducing impacts of PFA and PRO were 87% and 75% in non-transformed CP counts, respectively, when compared to NEG.

Table 4. Body weight gain, feed conversion ratio and *Clostridium perfringens* counts for negative control, narasin and classes of alternatives to antibiotics [1].

Class	Days 0–14		Days 14–28		Days 0–28		CP Counts log_{10} cfu/g
	BWG g	FCR g/g	BWG g	FCR g/g	BWG g	FCR g/g	
NEG [2]	474 ± 4	1.098 ± 0.006	1240 ± 9	1.338 ± 0.005	1714 ± 11	1.248 ± 0.003	6.09 ± 0.14
NAR [3]	488 ± 6 $p = 0.032$	1.064 ± 0.008 $p < 0.001$	1337 ± 12 $p < 0.001$	1.273 ± 0.007 $p < 0.001$	1825 ± 16 $p < 0.001$	1.192 ± 0.005 $p < 0.001$	2.92 ± 0.20 $p < 0.001$
PRO [4]	455 ± 8 $p = 0.012$	1.113 ± 0.009 $p = 0.118$	1283 ± 15 $p = 0.004$	1.302 ± 0.009 $p < 0.001$	1736 ± 19 $p = 0.239$	1.232 ± 0.006 $p = 0.004$	5.49 ± 0.25 $p = 0.017$
PRE [5]	479 ± 7 $p = 0.496$	1.095 ± 0.009 $p = 0.761$	1288 ± 14 $p < 0.001$	1.305 ± 0.008 $p < 0.001$	1767 ± 18 $p = 0.003$	1.229 ± 0.005 $p < 0.001$	5.70 ± 0.23 $p = 0.092$
PFA [5]	480 ± 7 $p = 0.375$	1.086 ± 0.009 $p = 0.152$	1247 ± 14 $p = 0.610$	1.323 ± 0.008 $p = 0.062$	1727 ± 17 $p = 0.457$	1.233 ± 0.005 $p = 0.004$	5.18 ± 0.23 $p < 0.001$
OA [5]	490 ± 7 $p = 0.025$	1.062 ± 0.009 $p < 0.001$	1288 ± 14 $p < 0.001$	1.325 ± 0.008 $p = 0.114$	1778 ± 17 $p < 0.001$	1.232 ± 0.005 $p = 0.002$	5.74 ± 0.23 $p = 0.130$
MIX [6]	479 ± 6 $p = 0.339$	1.087 ± 0.007 $p = 0.103$	1275 ± 11 $p = 0.002$	1.320 ± 0.007 $p = 0.007$	1754 ± 14 $p = 0.005$	1.234 ± 0.004 $p = 0.001$	5.79 ± 0.19 $p = 0.113$
ICC [7]	0.35	0.61	0.43	0.35	0.42	0.28	0.08

[1] Results are reported as means ± standard deviation. Body weight gain (BWG) in grams/chicken, feed conversion ratio (FCR) in grams feed intake/grams weight gain and *Clostridium perfringens* (CP) counts as log_{10} colony forming units/gram cecal content. [2] Negative control (no feed additive); production performance data based on $n = 66$ pens, and CP data based on $n = 198$ individual chicken samples. [3] Narasin; production performance data based on $n = 66$ pens, and CP data based on $n = 198$ individual chicken samples. [4] Probiotics (PRO); production performance data based on $n = 33$ pens, and CP data based on $n = 99$ individual chicken samples. [5] Prebiotics (PRE), phytogenics (PFA), organic acids (OA); production performance data based on $n = 44$ pens, and CP data based on $n = 132$ individual chicken samples. [6] Mixed products (MIX), i.e., treatments based on more than one ATA class; production performance data based on $n = 99$ pens, and CP data based on $n = 297$ individual chicken samples.[7] Intraclass correlation coefficient.

Three ATA classes (PRO, PRE and MIX) improved $FCR_{14–28}$ (1.3%–2.7% improvement, $p < 0.01$), and four classes (PRO, PRE, OA and MIX) increased $BWG_{14–28}$ (2.8%–3.9% increase, $p < 0.01$). Accumulated feed conversion during days 0 to 28 ($FCR_{0–28}$) was improved by all ATA classes (1.1%–1.5%, $p < 0.01$). However, only the OA class improved feed conversion during days 0 to 14 ($FCR_{0–14}$) significantly (3.3%, $p < 0.001$). Narasin outperformed the ATA classes at all age intervals, except for body weight gain during days 0 to 14 ($BWG_{0–14}$) and $FCR_{0–14}$, where the OA class performed similarly.

3.3. Impact of Treatments on Intestinal CP Counts and Production Performance

Intestinal CP counts were significantly reduced ($p < 0.05$) by 8 out of 24 ATA treatments (ID 3, 5, 15, 16, 18, 20, 21 and 24) as shown in Table 5. Estimated reduction in non-transformed CP counts among these eight treatments ranged from 84% to 97% when compared to NEG. Phytogenic components were present in 5/8 treatments (ID 15, 18, 20, 21 and 24), prebiotic components in 3/8 treatments (ID 5, 16 and 24), probiotic components in 2/8 treatments (ID 3 and 5) and OA components were present in 2/8 treatments (ID 15 and 24). Mean log_{10} CP counts with 95% confidence interval for each ATA treatment are shown in Figure 1.

Table 5. Body weight gain, feed conversion ratio and *Clostridium perfringens* counts for negative control, narasin and alternatives to antibiotics treatments [1].

ID-Class	Days 0–14		Days 14–28		Days 0–28		CP Counts log_{10} cfu/g
	BWG g	FCR g/g	BWG g	FCR g/g	BWG g	FCR g/g	
0-NEG [2]	474 ± 4	1.098 ± 0.006	1240 ± 9	1.338 ± 0.005	1714 ± 11	1.248 ± 0.003	6.09 ± 0.14
1-NAR [3]	488 ± 6 $p = 0.032$	1.064 ± 0.008 $p < 0.001$	1337 ± 12 $p < 0.001$	1.273 ± 0.007 $p < 0.001$	1825 ± 16 $p < 0.001$	1.192 ± 0.005 $p < 0.001$	2.92 ± 0.20 $p < 0.001$
2-PRO [4]	452 ± 11 $p = 0.049$	1.118 ± 0.014 $p = 0.153$	1285 ± 22 $p = 0.044$	1.305 ± 0.012 $p = 0.007$	1735 ± 29 $p = 0.455$	1.236 ± 0.008 $p = 0.120$	5.46 ± 0.38 $p = 0.097$
3-PRO [4]	451 ± 11 $p = 0.044$	1.110 ± 0.014 $p = 0.383$	1273 ± 22 $p = 0.132$	1.307 ± 0.012 $p = 0.012$	1723 ± 29 $p = 0.740$	1.235 ± 0.008 $p = 0.094$	5.11 ± 0.38 $p = 0.010$
4-PRO [4]	462 ± 11 $p = 0.274$	1.111 ± 0.014 $p = 0.357$	1290 ± 22 $p = 0.024$	1.295 ± 0.012 $p < 0.001$	1751 ± 29 $p = 0.198$	1.224 ± 0.008 $p = 0.002$	5.90 ± 0.38 $p = 0.623$
5-MIX [4]	472 ± 11 $p = 0.872$	1.084 ± 0.014 $p = 0.304$	1268 ± 22 $p = 0.207$	1.329 ± 0.012 $p = 0.459$	1739 ± 29 $p = 0.378$	1.244 ± 0.008 $p = 0.579$	5.21 ± 0.38 $p = 0.021$
6-PRE [4]	476 ± 11 $p = 0.854$	1.112 ± 0.014 $p = 0.324$	1305 ± 22 $p = 0.004$	1.280 ± 0.012 $p < 0.001$	1782 ± 29 $p = 0.023$	1.216 ± 0.008 $p < 0.001$	5.98 ± 0.38 $p = 0.782$
7-PRE [4]	470 ± 11 $p = 0.731$	1.106 ± 0.014 $p = 0.544$	1311 ± 22 $p = 0.002$	1.269 ± 0.012 $p < 0.001$	1781 ± 29 $p = 0.018$	1.211 ± 0.008 $p < 0.001$	5.91 ± 0.38 $p = 0.637$
8-MIX [4]	469 ± 11 $p = 0.672$	1.093 ± 0.014 $p = 0.708$	1293 ± 22 $p = 0.016$	1.280 ± 0.012 $p < 0.001$	1763 ± 29 $p = 0.086$	1.208 ± 0.008 $p < 0.001$	6.05 ± 0.38 $p = 0.928$
9-PFA [4]	459 ± 11 $p = 0.178$	1.108 ± 0.014 $p = 0.480$	1288 ± 22 $p = 0.030$	1.284 ± 0.012 $p < 0.001$	1747 ± 29 $p = 0.243$	1.221 ± 0.008 $p < 0.001$	5.89 ± 0.38 $p = 0.600$
10-OA [4]	499 ± 11 $p = 0.029$	1.073 ± 0.014 $p = 0.070$	1280 ± 22 $p = 0.072$	1.327 ± 0.012 $p = 0.368$	1780 ± 29 $p = 0.021$	1.233 ± 0.008 $p = 0.051$	5.76 ± 0.38 $p = 0.395$
11-MIX [4]	511 ± 11 $p = 0.001$	1.037 ± 0.014 $p < 0.001$	1335 ± 22 $p < 0.001$	1.317 ± 0.012 $p = 0.092$	1847 ± 29 $p < 0.001$	1.215 ± 0.008 $p < 0.001$	6.22 ± 0.38 $p = 0.720$
12-OA [4]	494 ± 11 $p = 0.078$	1.038 ± 0.014 $p < 0.001$	1287 ± 22 $p = 0.034$	1.324 ± 0.012 $p = 0.252$	1782 ± 29 $p = 0.017$	1.223 ± 0.008 $p = 0.001$	5.62 ± 0.38 $p = 0.222$
13-OA [4]	501 ± 11 $p = 0.019$	1.028 ± 0.014 $p < 0.001$	1318 ± 22 $p < 0.001$	1.311 ± 0.012 $p = 0.031$	1820 ± 29 $p < 0.001$	1.208 ± 0.008 $p < 0.001$	6.05 ± 0.38 $p = 0.918$
14-OA [4]	465 ± 11 $p = 0.423$	1.108 ± 0.014 $p = 0.469$	1266 ± 22 $p = 0.237$	1.340 ± 0.012 $p = 0.884$	1730 ± 29 $p = 0.567$	1.263 ± 0.008 $p = 0.058$	5.54 ± 0.38 $p = 0.147$
15-MIX [4]	476 ± 11 $p = 0.845$	1.097 ± 0.014 $p = 0.939$	1278 ± 22 $p = 0.085$	1.338 ± 0.012 $p = 0.977$	1754 ± 29 $p = 0.165$	1.255 ± 0.008 $p = 0.344$	5.18 ± 0.38 $p = 0.017$
16-PRE [4]	485 ± 11 $p = 0.352$	1.085 ± 0.014 $p = 0.346$	1304 ± 22 $p = 0.004$	1.335 ± 0.012 $p = 0.822$	1788 ± 29 $p = 0.009$	1.251 ± 0.008 $p = 0.669$	4.76 ± 0.38 $p < 0.001$
17-MIX [4]	458 ± 11 $p = 0.157$	1.105 ± 0.014 $p = 0.588$	1228 ± 22 $p = 0.593$	1.354 ± 0.012 $p = 0.185$	1685 ± 29 $p = 0.316$	1.270 ± 0.008 $p = 0.004$	6.33 ± 0.38 $p = 0.518$
18-PFA [4]	491 ± 11 $p = 0.132$	1.067 ± 0.014 $p = 0.025$	1226 ± 22 $p = 0.524$	1.353 ± 0.012 $p = 0.216$	1717 ± 29 $p = 0.926$	1.243 ± 0.008 $p = 0.552$	5.31 ± 0.38 $p = 0.040$
19-PRE [4]	485 ± 11 $p = 0.371$	1.078 ± 0.014 $p = 0.158$	1229 ± 22 $p = 0.624$	1.336 ± 0.012 $p = 0.850$	1713 ± 29 $p = 0.971$	1.237 ± 0.008 $p = 0.156$	6.12 ± 0.38 $p = 0.918$
20-PFA [4]	486 ± 11 $p = 0.301$	1.091 ± 0.014 $p = 0.590$	1228 ± 22 $p = 0.592$	1.334 ± 0.012 $p = 0.748$	1713 ± 29 $p = 0.987$	1.242 ± 0.008 $p = 0.428$	4.95 ± 0.38 $p = 0.003$
21-PFA [4]	485 ± 11 $p = 0.330$	1.077 ± 0.014 $p = 0.128$	1246 ± 22 $p = 0.799$	1.321 ± 0.012 $p = 0.170$	1730 ± 29 $p = 0.566$	1.226 ± 0.008 $p = 0.004$	4.59 ± 0.38 $p < 0.001$
22-MIX [4]	486 ± 11 $p = 0.301$	1.089 ± 0.014 $p = 0.502$	1270 ± 22 $p = 0.179$	1.320 ± 0.012 $p = 0.146$	1755 ± 29 $p = 0.147$	1.231 ± 0.008 $p = 0.028$	5.56 ± 0.38 $p = 0.168$
23-MIX [4]	484 ± 11 $p = 0.355$	1.083 ± 0.014 $p = 0.273$	1251 ± 22 $p = 0.612$	1.327 ± 0.012 $p = 0.378$	1736 ± 29 $p = 0.448$	1.235 ± 0.008 $p = 0.090$	5.94 ± 0.38 $p = 0.694$
24-MIX [4]	494 ± 11 $p = 0.078$	1.086 ± 0.014 $p = 0.386$	1292 ± 22 $p = 0.018$	1.307 ± 0.012 $p = 0.014$	1786 ± 29 $p = 0.011$	1.222 ± 0.008 $p < 0.001$	5.30 ± 0.38 $p = 0.037$
25-MIX [4]	464 ± 11 $p = 0.394$	1.105 ± 0.014 $p = 0.610$	1255 ± 22 $p = 0.489$	1.311 ± 0.012 $p = 0.028$	1720 ± 29 $p = 0.844$	1.229 ± 0.008 $p = 0.013$	6.33 ± 0.38 $p = 0.518$
ICC [5]	0.35	0.61	0.43	0.35	0.42	0.28	0.08

[1] Results are reported as means ± standard deviation. Body weight gain (BWG) in grams/chicken, feed conversion ratio (FCR) in grams feed intake/grams weight gain and *Clostridium perfringens* (CP) counts as log_{10} colony forming units/gram cecal content. [2] Negative control (no feed additive); production performance data based on $n = 66$ pens, and CP data based on $n = 198$ individual chicken samples. [3] Narasin; production performance data based on $n = 66$ pens, and CP data based on $n = 198$ individual chicken samples. [4] Probiotics (PRO), prebiotics (PRE), phytogenics (PFA), organic acids (OA), mixed products (MIX); production performance data based on $n = 11$ pens, and CP data based on $n = 33$ individual chicken samples. [5] Intraclass correlation coefficient.

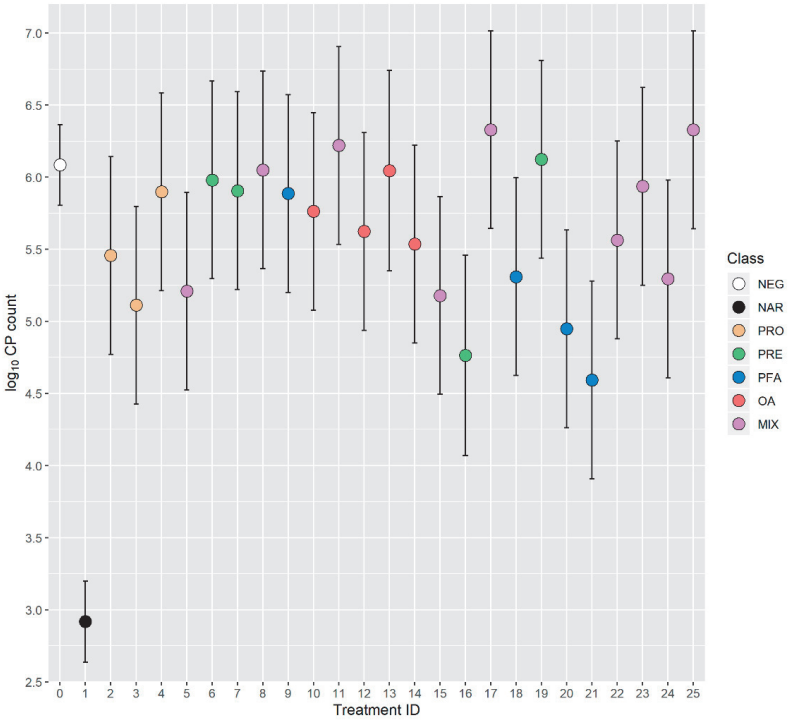

Figure 1. Cecal *Clostridium perfringens* (CP) counts with 95% confidence intervals. Negative control (NEG) is treatment 0, narasin (NAR) is treatment 1, probiotics (PRO) are treatments 2–4, prebiotics (PRE) are treatments 6, 7, 16 and 19, phytogenics (PFA) are treatments 9, 18, 20 and 21, organic acids (OA) are treatments 10, 12, 13 and 14, and mixed products (MIX) are treatments 5, 8, 11, 15, 17 and 22–25.

$FCR_{14–28}$ was improved ($p < 0.05$) by 10/24 tested ATA treatments (Table 5). Five of these treatments (ID 4, 6, 7, 8 and 9) achieved $FCR_{14–28}$ improvements (3.2% to 5.2%, $p < 0.001$) that returned the same significance level as narasin (4.9% improvement, $p < 0.001$). These five treatments had active components classified as probiotics (ID 4), prebiotics (ID 6 and 7), phytogenics (ID 8 and 9) or organic acids (ID 8). In total, 13/24 ATA treatments improved $FCR_{0–28}$ (1.4% to 3.2% improvement, $p < 0.05$). Seven of these treatments (ID 6, 7, 8, 9, 11, 13 and 24) achieved improvements in FCR $_{0–28}$ that returned the same significance level (2.1% to 3.2% improvement, $p < 0.001$) as narasin (4.5% improvement, $p < 0.001$).

$BWG_{14–28}$ and body weight gain during days 0 to 28 ($BWG_{0–28}$) were increased by 10/24 and 8/24 ATA treatments, respectively. Two treatments (ID 11 and 13) excelled in increasing both these parameters, with a significance level similar to narasin ($p < 0.001$).

In the sixth trial, two-product combinations of treatments with predominantly CP-reducing impact (ID 5, 16 and 21) and treatments with predominantly production performance-promoting impact (ID 7, 11 and 13) were evaluated (comprising treatment ID 22–25 in Table 2). Treatment 16 did not appear to reduce the FCR-improving effect of treatments 11 and 13 (Table 5) but tended to diminish the growth promoting impact of these treatments. Treatment 5 seemed to diminish the FCR-improving effect and remove the growth-promoting effect of treatment 7. Treatment 21 appeared to reduce or remove the improvement in FCR and to remove the growth-promoting effect of treatment 7. On the other hand, treatment 7 seemed to remove the CP-reducing impact of treatments 5 and 21, and treatment 13 appeared to remove the CP-reducing impact of treatment 16. In contrast to these

results, treatment 11 did not appear to impair the CP-reducing impact of treatment 16. As a result of these interactions between predominantly CP-reducing and production performance-promoting single treatments, treatment 24 was the only one of four tested product combinations (ID 22–25, Table 5) with beneficial effects on production performance variables as well as CP counts.

3.4. Active Components with Combined Beneficial Effects on $FCR_{14–28}$ and CP Counts

In total, 10/24 and 8/24 tested treatments improved ($p < 0.05$) $FCR_{14–28}$ and CP counts, respectively. Collectively these treatments comprised a group of 16 treatments; 14 treatments either improved $FCR_{14–28}$ or reduced CP counts, and only two treatments (ID 3 and 24) influenced both $FCR_{14–28}$ and CP counts in a beneficial way. One of the two superior treatments according to these criteria was a probiotic (ID 3) with the *Bacillus subtilis* PB6 strain as the only active component. The other treatment (ID 24) was a combination of three ATA classes (OA/PFA/PRE) and two products; one product (ID 16) containing whole cells of *Saccharomyces cerevisiae* and its metabolites, and another (ID 11) being a mixture of SCFAs (including C4), MCFAs (including C12) and a phenolic compound.

4. Discussion

The collective group of 24 ATA treatments tested in this study reduced the occurrence of NE and reduced intestinal CP counts after *Eimeria* challenge. Production performance measured as BWG and FCR was improved by the collective ATA group, but not significantly during the phase prior to *Eimeria* challenge. These results indicate a beneficial effect of several ATAs when chickens are exposed to mild to moderate intestinal stress. The favorable effects of the polyether ionophore narasin on CP counts and production performance were numerically and in part significantly stronger than the effect of the collective group of ATA treatments.

In this study, the chickens were challenged orally with five precocious lines of *Eimeria* spp. as a predisposing factor for NE. NE is expected to appear during oocyst excretion, which begins between three and four days following inoculation with precocious *Eimeria acervulina* [37] and *Eimeria mitis* lines, and presumably later with precocious *Eimeria maxima* and *Eimeria tenella* lines [38]. In a previous study with a similar type of *Eimeria* challenge, most gut damage was detected four to six days after inoculation [39]. Postmortem examinations in this study confirmed the presence of NE during this time span after *Eimeria* challenge. Intestinal CP counts are strongly associated with NE [19,34,40]. Furthermore, increased occurrence of NE in commercial broiler flocks has been associated with impaired accumulated FCR at slaughter [20]. Weakened production performance is likely to be most pronounced during the part of the rearing phase that is affected by NE. Based on the considerations mentioned above we chose to emphasize CP counts on days four to six after *Eimeria* challenge and $FCR_{14–28}$ in our analyses of effect of ATAs on intestinal health.

The evaluation of ATAs can rest on different criteria, depending on point of view, practical circumstances and current health problems. Disease conditions associated with increased metabolism and rapid growth in broilers (in particular cardiovascular and musculoskeletal disorders) are generally important, and it has been claimed that such conditions cause greater economic loss than infectious agents [41]. In a recent study, it was found that chickens with higher body weight and BWG were predisposed to develop more severe NE lesions when challenged with CP [42]. Although attractive in the short run, increased weight gain may therefore come at a cost not only to chicken health and welfare but also to the farmers' economy and a sustainable use of feed resources. In light of this consideration, we have emphasized the effect on FCR as production performance parameter, because it is an indicator of intestinal health as well as resource efficiency.

The only two ATA treatments (ID 3 and 24) with a combined beneficial effect on CP counts (84 to 89% reduction, $p < 0.05$) and $FCR_{14–28}$ (2.3% improvement, $p < 0.05$) were based on different types of active components. One of the treatments (ID 3) was a mono-strain (*Bacillus subtilis* strain PB6) spore-forming bacterial probiotic. This probiotic strain has been reported to inhibit CP in vitro [43] and improve FCR [44], which is in agreement with our findings.

The other treatment (ID 24) was based on a heterogeneous collection of active components including short- and medium-chain fatty acids, a phenolic compound and dehydrated whole cells and metabolites of the yeast *Saccharomyces cerevisiae* (SC). This treatment comprised two commercial products that were also tested individually (ID 11 and 16). Whereas the yeast product (ID 16) alone demonstrated a 95% reduction ($p < 0.001$) in non-transformed CP counts, the product containing a blend of organic acids and a phenolic compound (ID 11) had no reducing impact on CP. Viewed against this background it seems probable that the CP reducing effect of treatment 24 was mainly associated with one or several yeast components found in treatment 16. In addition to treatment 24, three other ATA treatments based on the yeast SC were tested. These treatments, which were based on SC cell wall extracts (ID 6, 7 and 19), did not reduce CP counts to an extent that was significant with the sample size and/or feed additive dosage used in our study, whilst the treatments based on SC whole cells and metabolites (ID 16 and 24) did. No previous reports on the effect of SC metabolites and SC whole cells on CP counts in broilers have been found. Regarding yeast cell wall extracts, previously published literature has indicated both significant [45] and non-significant [46] CP-reducing impact. Our results indicate that whole cells and/or metabolites of SC inhibited intestinal CP growth more efficiently than SC cell wall extracts with the product inclusion levels used in this study.

One of the active components in treatment 11 was lauric acid (C12), a MCFA that has been demonstrated to inhibit CP in vitro [47]. Treatment 11 did, as mentioned above, not reduce CP counts when used as sole feed additive in this study. Possible explanations include too low concentration of lauric acid and/or interfering effects by other treatment components.

Regarding production performance, the combination of treatments 11 and 16 (i.e., treatment 24) had a significantly beneficial effect on $FCR_{14–28}$. However, neither of these two treatments improved $FCR_{14–28}$ when tested individually. This finding suggests a synergy effect with regard to $FCR_{14–28}$ between active components present in the two products. Beneficial effects of dietary supplementation of whole cells and metabolites of SC on production performance in broilers have been reported by others [48].

The combination of SCFAs (including butyric acid-C4), MCFAs (including lauric acid-C12) and a phenolic compound in treatment 11 generated the numerically highest weight gain ($BWG_{0–14}$ and $BWG_{14–28}$) of all ATA treatments in this study but had no apparent impact on CP counts. This result suggests that rapid growth is possible in the presence of relatively high cecal CP counts. A possible explanation could be that this treatment reduced the counts of virulent CP strains (e.g., strains harbouring the *netB* gene) or the expression of virulence factors (e.g., the NetB toxin), but not the total CP counts. Treatment 11 might also have influenced the intestinal microbiota in a way that neutralized the negative impact of high CP counts.

Six of 24 ATA treatments (ID 5, 15, 16, 18, 20 and 21) were associated with reduced CP counts (at least 83% reduction in non-transformed counts, $p < 0.05$) without improving $FCR_{14–28}$ significantly. Treatment 21 had a very strong reducing impact on CP counts (a 97% reduction, $p < 0.001$) and improved $FCR_{0–28}$ (1.8%, $p < 0.01$), but had only a numerically (1.3%, non-significant) beneficial impact on $FCR_{14–28}$. Active components of treatment 21 included oleoresins from turmeric (*Curcuma longa*) and chili peppers (genus *Capsicum*). These results are in agreement with reports on inhibitory activity against CP of turmeric extracts [49], reduced gut lesion scores in CP-challenged broilers treated with *Capsicum* and *Curcuma* oleoresins [50,51] and improved cumulative FCR of turmeric powder [52]. Treatment 20 was based on tall oil fatty acids from coniferous trees including resin acids. Resin acids have been reported to inhibit CP in vitro [53], and our results suggest similar effects in vivo.

Treatments 22–25 were tested in a final trial intended to evaluate two-product combinations of treatments improving production performance and treatments with CP-reducing impact. Treatment 24 was the only combination with beneficial impact on both CP counts and production results. These results suggest that the interaction between predominantly CP-reducing and production-promoting components vary substantially. Among three tested CP-reducing treatments in the final trial (ID 5, 16 and 21), a treatment based on dehydrated SC culture with whole cells and metabolites (ID 16)

was the least impairing with regard to the production-promoting effects of its combination treatment. Among the three tested production performance-improving treatments (ID 7, 11 and 13), treatment 11 based on short- and medium-chain fatty acids and other components was the only one that did not impair the CP-reducing impact of its combination treatment. More work is needed to identify the role of the different components in treatments 11 and 16, and whether the beneficial interaction of these components also can be extended to include other CP-reducing and production-promoting components.

Our findings indicate that a reduction of CP counts induced by ATAs was not always associated with improved production performance. Lack of a positive impact on feed efficiency and growth rate has also been documented with regard to ionophores under certain conditions [54], in spite of these compounds' suppressing effect on CP counts. However, when used at recommended concentrations in broiler flocks exposed to coccidia, the net effect of ionophores is usually improved performance. In our study, considerably improved performance combined with a strong CP-reducing effect of the ionophore narasin was present. These results confirm that our challenge model worked as expected, and that the in-feed concentration of narasin was within the optimal range. The reason why some of the ATAs with CP-reducing effect in our study did not induce a significantly positive net impact on production performance under the same test conditions remains unclear. Possible explanations may be that the inhibiting effect on CP was accompanied by reduced ability to utilize feed efficiently and/or establishment of another performance-impairing intestinal microbiota.

Eight of 24 ATA treatments (ID 2, 4, 6, 7, 8, 9, 13 and 25) improved $FCR_{14–28}$ (at least 2.0% improvement, $p < 0.05$) without reducing CP counts significantly. One of these treatments (ID 4) was a mono-strain *Bacillus subtilis* probiotic. Data from other studies demonstrate the capacity of *Bacillus subtilis* strains to suppress the growth of CP and improve production performance and intestinal morphology [55–57]. However, the favorable impact on $FCR_{14–28}$ in this study might have been caused by other mechanisms than inhibition of CP growth. Suggested modes of action associated with probiotics are maintenance of balanced microbial populations, modulation of the host immune system, promotion of epithelial barrier integrity and alteration of villus length and crypt depth [44,58–61].

Two (ID 6 and 7) of the treatments improving $FCR_{14–28}$ but not CP counts contained cell wall extracts from the yeast SC. Both treatments 6 and 7 had a considerably beneficial impact on $FCR_{14–28}$ (estimated 4.3 and 5.2% reduction, respectively). Of the SC cell wall-based treatments, the products with the apparently highest content of β-glucans (ID 6 and 7) had the best effect on $FCR_{14–28}$ as compared with the other yeast cell wall-based treatment in our study (ID 19). Treatment 7, containing minimum 60% purified β-1.3/1.6 glucans, even outperformed narasin numerically with regard to $FCR_{14–28}$. These findings suggest that SC-derived β-glucans are potent when it comes to improvement of FCR in broilers exposed to *Eimeria* spp. Beneficial effects of yeast β-glucans on performance in broilers are supported by some [62,63] and in contradiction with results from other previous reports [64,65]. Possible explanations for the $FCR_{14–28}$-promoting effect of feed additives containing β-glucans are modulations of the immune response [62,64].

Two other treatments (ID 8 and 9) with favorable effect on $FCR_{14–28}$ without significant reduction of CP counts contained essential plant oils. Essential oil components in treatments 8 and 9 included thymol (in both treatments), eugenol and piperine (in treatment 8) and carvacrol, anethol and limonene (in treatment 9). In treatment 8, essential oils were combined with benzoic acid. Published results on effects and mode of action of essential oils suggest that several of these compounds inhibit the growth of CP [66–68], although the findings are not always clear cut [69], or they show no effect on CP counts [70]. Reports on mitigation of gut lesions in chickens challenged with CP [67,71] underpin the view that at least thymol and carvacrol suppress the pathogenic action of CP. Studies on the effect of essential oil components on production performance reveal variable results. One study reports a negative effect on broiler performance using a blend of thymol, eugenol, curcumin and piperine [70], another describe a non-significant tendency of improved $FCR_{14–28}$ using a blend of carvacrol and thymol [71], and a third study presents significant improvement of $FCR_{0–28}$ by carvacrol but not by thymol [72]. The lack of standardization of studies, including variable feed additive dosage and different combinations of

active compounds, makes comparison of results from different studies difficult. The interpretation of results is further complicated by the multiple suggested effects of different essential oils, including antibacterial and antioxidant properties, enhancement of the immune system, and stimulation of digestive secretions and blood circulation [73,74]. Regardless of mechanism, these two predominantly phytogenic feed additives (ID 8 and 9) had a pronounced beneficial effect on $FCR_{14–28}$ on par with narasin in the current study.

The apparent lack of a CP-reducing effect of yeast cell wall extracts, essential oils and other active components associated with improved $FCR_{14–28}$ in this study may in part be related to experimental design. As observed from our results (Table 5), estimated CP count reductions of 76% or less (e.g., treatment ID 2 and 14) returned non-significant (>0.05) *p*-values. The main reason for this low statistical power was high variance of CP counts in individual observations within each treatment, leading to imprecise estimates. Our experiments were designed with 33 replicates of individual CP counts per ATA treatment, and this sample size returned relatively wide confidence intervals (as shown in Figure 1). The statistical analysis involving the whole ATA group (Table 3) indicated that when 792 individual samples with a $\log_{10}$ 5.63 CP estimate were compared with the NEG group with 198 individual samples and a $\log_{10}$ 6.09 estimate (corresponding to a 65% difference), this difference was significant ($p = 0.005$).

The ATAs did not suppress CP counts to the same extent as the ionophorous coccidiostat narasin. The superior results of narasin in this respect were most likely due to the strong antibacterial effect of this compound. Narasin has been reported with inhibitory effect on CP growth similar to or better than antibiotics used as drinking water medication for poultry [75].

Different ATAs can add value to the broiler chicken industry in several ways. Some improve BWG and/or FCR, others inhibit growth of CP or have a beneficial effect on both production performance parameters and intestinal CP counts. The use of specific ATAs could possibly be targeted to specific age intervals or current health status in the flock. Future studies of the impact of ATAs on intestinal CP counts would most likely benefit from modified sampling protocols and quantification methods. Study designs that were useful for investigating the effect of AGPs and ionophorous coccidiostats should not be copied without reservation when studying non-antibiotic alternative feed additives. Finally, a less pronounced effect than narasin of selected ATAs on production performance and/or CP counts in this study does not necessarily mean that the impact is of no importance to broiler health and production economy.

In this study, ATA classes displayed distinct performance profiles. The probiotic class reduced CP counts and improved production performance during the time period with intestinal stress (days 14–28), but impaired weight gain during days 0–14. The prebiotic class improved production performance during days 14–28 and had a non-significantly reducing impact on CP counts. The phytogenic class had a markedly reducing impact on CP counts and improved $FCR_{0–28}$. The organic acid class increased weight gain throughout the study period and improved $FCR_{0–14}$ but did not reduce CP counts significantly. These findings suggest that employing ATA classes for specific purposes may be useful. As an example, combining probiotic and organic acid treatments might boost production performance throughout the grow-out period and at the same time reduce CP counts during intestinal stress. In this study, we tested other ATA class combinations with variable results, indicating the need for testing of specific combinations of active components within the ATA classes.

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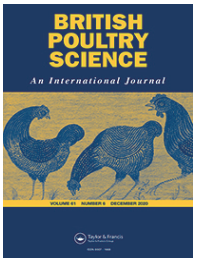
Paper II



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Varying starch to fat ratios in pelleted diets: I. Effects on nutrient digestibility and production performance in *Eimeria*–challenged broiler chickens

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Varying starch to fat ratios in pelleted diets: I. Effects on nutrient digestibility and production performance in *Eimeria*–challenged broiler chickens

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ABSTRACT

1. The hypothesis was that a diet with a high starch to fat ratio (HS) impairs nutrient digestibility and growth performance, as compared to a diet with a low starch to fat ratio (LS) in *Eimeria*–challenged broilers. From days 10 to 29, 12 replicate pens of birds were given isocaloric and isonitrogenous steam-pelleted diets with either HS or LS, by replacing the wheat starch in one diet by a mixture of rapeseed oil and inert sand in the other. On d 17, a 10-fold dose of live vaccine strains of *Eimeria spp*. was administered *via* drinking water. Ileal samples were collected on days 16 and 29.
2. Starch content in the ileum tended to be higher on d 16 and was significantly higher on d 29 in the HS group.
3. The HS diet did not induce exceedingly high levels of starch in the ileum, suggesting there was no starch overload in the gut. Ileal starch digestibility was improved with increasing dietary starch level from 23% to 45%. This demonstrated the capacity of the broiler chicken to digest high levels of starch regardless of *Eimeria spp*. infection. Ileal energy digestibility was not affected by the treatments.
4. Weight gain did not differ between treatments; however, birds fed the LS diet were less efficient in feed conversion as compared to those fed the HS diet.
5. The use of isolated starch and the unintended higher extent of starch gelatinisation in the HS diet may have contributed to the higher starch digestibility in birds given the HS diet. Thus, the hypothesis that high ratios of starch to fat in pelleted diets may impair starch digestibility and production performance in *Eimeria*-challenged broiler chickens was not verified. Further work is required to clarify this research question, taking into consideration the physical form of starch source and the potentially confounding role of feed processing on starch availability.

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Introduction

Broiler chickens are efficient at utilising starch as their main energy source (Thomas et al. 2008). This ability is presumably due to sufficient amylase secretion (Svihus 2014), high activity levels of disaccharidases shortly after hatching (Chotinsky et al. 2001) and a highly adaptive intestinal mechanism for glucose uptake (Suvarna et al. 2005). Nevertheless, starch digestibility has been observed to be low in broilers given wheat-based pelleted diets, with values ranging from 0.76 to 0.93 (Svihus 2001; Svihus et al. 2010; Abdollahi et al. 2011). Svihus and Hetland (2001) evaluated starch digestibility in broiler chickens fed identical wheat diets that were pelleted (control), offered as mash or pelleted and diluted with 100 g/kg cellulose powder. Compared to the mash diet and the diluted diet, undiluted pelleting resulted in an overload of wheat-starch (reaching more than 200 g/kg freeze-dried ileal contents) in ileal chyme and consequently poorer starch digestibility. Accordingly, the authors proposed that reducing dietary starch level (diet dilution) or a decrease in feed intake (by changing diet structure) may be potential means to prevent excessive concentration of starch in the ileum, thereby optimising digestion.

The physiological ability to digest lipids is not fully developed in the young chick due to low lipase activity (Krogdahl 1985; Noy and Sklan 1995) and insufficient bile acid secretion (Sell 1996). More recently, Tancharoenrat et al. (2013) confirmed this limited capacity in one-week-old chicks, and detected a significant increase in total tract digestibility (from 0.53 to 0.81) of fat at two-weeks of age independent of fat type.

Increased amounts of undigested nutrients in the digestive tract may stimulate undesirable microbial growth that could induce enteric disorders (Choct et al. 1999; Annett et al. 2002). Corroborating this, Engberg et al. (2004) found a tendency for increased ileal and caecal numbers of *Clostridium perfringens* due to the presence of more starch and other fermentable nutrients in the small intestine of broilers fed a pelleted wheat diet. *Eimeria spp*. infection is another factor that may lead to microbial and intestinal dysfunctions (Yun et al. 2000; Hauck 2017), and consequently increase broiler intestinal vulnerability to other types of intestinal insults and imbalances.

Starch is the major energy-supplying source in broiler diets, but when prices are favourable, it may be preferred to replace starch with fat in the diet. Due to the rising prices of cereal grains, the use of grain-replacing, unconventional feedstuffs is increasing, and so more fat is added to increase dietary energy content. The effect of varying dietary starch to fat ratios on the performance of broilers fed isocaloric and isonitrogenous diets have been investigated and produced inconsistent results. For Veldkamp et al. (2017a), Veldkamp et al. (2017b) reported an improvement in feed conversion ratio (FCR) and growth performance with higher starch to

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fat ratio. Malheiros et al. (2004) on the other hand reported slightly better FCR with lower starch to fat ratio, whereas Baéza et al. (2015) found that performance parameters were not affected by the varying ratios of starch to fat.

Thus, the hypothesis tested was that a diet with a high starch to fat ratio (31:1) would result in lower intestinal starch digestibility, increased concentrations of undigested starch in the posterior small intestine and impaired production performance in *Eimeria*–challenged broilers. The present paper focusses on nutrient digestibility and production performance, while effects on intestinal histomorphology, *C. perfringens* counts and toxin profile, necrotic enteritis prevalence and abundance of short-chain fatty acids are discussed in an accompanying paper (Granstad et al. in press).

Materials and methods

Experimental diets and processing

Experimental diets (Table 1) were processed at the Centre for Feed Technology (Fôrtek), Norwegian University of Life Sciences, Ås, Norway, and were formulated to meet or exceed Ross 308 strain recommendations for major nutrients (Aviagen 2014). The diets contained 5 g/kg titanium dioxide as a digestibility marker. The wheat and soybean meal (SBM) were ground to pass through a 3-mm sieve in a hammer mill (Münch-Edelstahl, Wuppertal, Germany licenced by Bliss, USA, 18.5 kW, 3000 RPM) before being mixed with other ingredients. The mash was steam-conditioned in a double pass pellet-press conditioner (Münch-Edelstahl, Wuppertal, Germany) and then pelleted using a pellet press (Münch-Edelstahl, Wuppertal, Germany, 1.2 t/h, 2 × 17 kW, RMP 350) equipped with a 60-mm-thick die with 5-mm diameter die openings. Conditioning temperature and production rates were 71°C and 700 kg/h for the diet with a high starch to fat ratio (HS), and 81°C and 800 kg/h for the diet with a low starch to fat ratio (LS). Specific energy consumption values were 45.7 and 18.5 kWh/t, and motor load was 52 and 24 A for the diet with a HS and LS, respectively. Despite the reduced conditioning temperature, post-pelleting temperatures were 95°C in the diet with a HS compared to 81.9°C for the diet with a LS, measured by collecting a sample of hot pellets from immediately below the pellet press into an insulated box fitted with a thermometer. The extent of starch gelatinisation was almost 7.3-fold higher with HS compared to LS (Table 1).

Table 1. Experimental diet composition, calculated and analysed nutrient content (g/kg as fed).

Ingredients	HS*	LS*
Wheat	412.6	412.6
Fish meal (72% CP)	100	100
Soybean meal (47.3% CP)	185	185
Wheat starch[1]	250	-
Rapeseed oil	-	87.4
Sand[2]	-	162.6
L-Lysine	2.8	2.8
DL-Methionine	2.8	2.8
L-Threonine	2	2
Limestone	12	12
Monocalcium phosphate	15	15
Sodium chloride	3	3
Titanium dioxide	5	5
Choline chloride	2	2
Mineral & Vitamin premix[3]	6.3	6.3
Enzyme (Rovabio)[4]	1.5	1.5
Calculated nutrient content		
Metabolisable energy (MJ/kg)	12.13	12.13
Dig. Lysine	12.9	12.9
Dig. Methionine	6.1	6.1
Dig Threonine	8.6	8.6
Analysed nutrient content		
Gross energy (MJ/kg)	16.20	15.95
DM (g/kg)	908	913
Starch (g/kg)	448	231
Fat (g/kg)	14.2	95.4
Crude Protein (g/kg)	211	211
Calcium (g/kg)	13.7	13.3
Phosphorous (g/kg)	8.1	7.9
Starch gelatinisation, g/kg starch	574.9	152.4
Starch: fat ratio	31.5: 1	2.4: 1

* HS and LS: high and low starch to fat ratio.

[1] Wheat starch, low gluten (produced by Roquette Amilina AB, provided by Alimenta AS, Hagan, Norway): Dry matter, 87%; Starch, 86%; Protein (Nx6.25), 0.35% max; Lipids, 0.1% max; Cellulose, 0.1% max and particle size distribution as follows: >200 µm, 2% max; >10 µm, 75% min.

[2] High purity quartz sand, NC4AF (The Quartz Corp, Drag, Norway): SiO_2 > 99.9%; particle size distribution as follows: >150 µm <5%; 75–150 µm >75%; <75 µm <15%.

[3] Mineral and vitamin premix provided the following per kg diet: Fe, 53 mg; Mn, 125 mg; Zn, 83 mg; Cu, 15 mg; I, 0 · 75 mg; Se, 0 · 30 mg; retinyl acetate, 5.75 mg; cholecalciferol, 0.18 mg; dl-α-tocopheryl acetate, 80 mg; menadione, 10 mg; thiamine, 6 mg; riboflavin, 26 mg; niacin, 35 mg; calcium pantothenate, 26 mg; pyridoxine, 15 mg; cobalamin, 0.04 mg; biotin, 0.6 mg; folic acid, 5 mg.

[4] Enzyme Rovabio Excel AP T-Flex (Adisseo, Antony, France) provided the following per kg diet: Endo-1,4-β-xylanase: 33 000 visco units; Endo-1,3(4)-β-glucanase: 45 000 visco units; Endo-1,4-β-glucanase (cellulase) >9600 DNS units + 16 other enzyme activities obtained from a fermentation broth of *Penicillium funiculosum*.

Birds and housing

The experiment was approved by the national animal research authority (Norwegian Food Safety Authority, approval ID 8824) and performed in accordance with national and international guidelines for the care and use of experimental animals.

A total of 1920 one-day-old mixed-sex Ross 308 broiler chicks obtained from a commercial hatchery (Nortura Samvirkekylling, Våler, Norway) were placed in 24-floor pens measuring 5.6 m^2 with new wood shavings. Each pen housed 80 feather-sexed birds with a 50/50 male-female distribution. A room temperature of 33°C was maintained during the first week and thereafter decreased by 3–4°C weekly until the temperature reached 21°C. Water and feed were given *ad libitum*. The birds were exposed to 23 h light per day on the first 2 days. For the rest of the experimental period, the birds were exposed to 16 h light per day, interrupted by two, 4 h periods of darkness. All birds were fed a commercial starter diet from 0 to 9 d of age. From d 10 to day 29, the birds were randomly divided into two groups of 12 pens each and fed either an HS or an LS grower diet.

Eimeria *challenge*

A 10-fold dose of the vaccine Paracox-5 vet. (MSD Animal Health, Boxmeer, the Netherlands) containing live, sporulated oocysts from five attenuated strains of *Eimeria spp*. (one strain each of *E. acervulina*, *E. mitis* and *E. tenella* and two strains of *E. maxima*) was administered via the drinking water of all birds on d 17 post hatch.

Production performance measurements

The amount of feed per pen was weighed when allocated, and feed residues were weighed before being discarded at feed change and at the end of the experiment. Accumulated feed

intake (FI) per pen from days 10–15, 15–24, 24–28 and 10–28 was calculated. Total live chicken weight per pen was recorded on d 10, 15, 24 and 28, and mean body weight gain (BWG, g/bird) and mean feed conversion ratio (FCR, g feed intake/g weight gain) per pen were calculated.

Sample collection

On days 16 and 29, two birds per pen were randomly selected and killed by a cranial blow followed by cervical dislocation. The small intestine with content was removed and placed in a zigzag pattern over an aluminium foil on a rack, and then immediately snap-frozen with liquid nitrogen and stored at −20°C for later analysis. A section from the posterior jejunum with content (5 cm anterior to Meckel's diverticulum) was later removed and stored at −80°C until enzyme activity analysis. The jejunum was defined as the segment from the end of the duodenal loop to Meckel's diverticulum, and the ileum as the section from Meckel's diverticulum to the ileocaecal junction.

Chemical analyses

Representative feed samples were ground on a cutting mill (Pulverisette 19, Fritsch Industriestr. 8, 55743 Idar-Oberstein, Germany) through a 0.5 mm sieve. Dry matter and ash content of the feed and ileal samples were determined after drying overnight at 105°C and after 12 h ashing at 550°C, respectively. Gross energy was determined using an adiabatic bomb calorimeter (Parr 6400, Moline, USA) standardised with benzoic acid. Nitrogen content was determined by the Dumas method using a Vario El Cube (Elementar Analysensysteme GmbH, Hanau, Germany 2016). Dried ileal contents were pulverised using a mortar and pestle for subsequent starch, crude fat, gross energy and titanium dioxide analysis. TiO_2 content of feed and ileal contents was determined as described by Short et al. (1996). Crude fat was determined after extraction with 80% petroleum ether and 20% acetone in an Accelerated Solvent Extractor from Dionex (ASE200; Sunnyvale, CA, USA). Starch content of the diets was determined enzymatically based on the use of thermostable α-amylase and amyloglucosidase (McCleary et al. 1994). Starch content in freeze-dried ileal samples was determined as described above after extraction with 80% ethanol (2x) to remove free sugars and oligosaccharides. Amylase activity in the jejunal chyme was assayed colorimetrically using amylase assay kit (Abcam ab102523, Cambridge, UK) according to manufacturer's instructions. Samples for amylase activity were prepared as described by Pérez de Nanclares et al. (2017) and results were expressed as unit/g of wet chyme. The degree of starch gelatinisation (DG) (as a proportion of total starch) was measured by differential scanning calorimetry (DSC 823e Module, Mettler-Toledo, Switzerland) as described by Kraugerud and Svihus (2011).

Calculations

The apparent ileal digestibility coefficients of starch, fat and energy were calculated using the following formula:

$$\text{Ileal digestibility coefficient} = \frac{\left(\frac{Nut}{Ti}\right)diet - \left(\frac{Nut}{Ti}\right)ileum}{\left(\frac{Nut}{Ti}\right)d\ iet}$$

where $\left(\frac{Nut}{Ti}\right)diet$= the ratio of nutrient and TiO_2 in the diet and $\left(\frac{Nut}{Ti}\right)ileum$= the ratio of nutrient and TiO_2 in the ileal digesta.

Statistical analysis

Statistical analyses were carried out using the statistical software R (version 2.3.2). All data sets were tested for normality using the Shapiro–Wilk test. A non-normal distribution of production performance data, nutrient content in ileal digesta, nutrient digestibility and amylase activity precluded the use of a parametric statistical test and hence these variables were compared using the two-way Wilcoxon rank-sum test (non-parametric). Differences were considered significant at $P < 0.05$ and results were expressed as means ± standard error. Each pen was used as the experimental unit for all data.

Results

Production performance

From d 10 to 15, no significant differences in feed intake (FI), body weight gain (BWG) and/or feed conversion ratio (FCR) were observed between dietary treatments (Table 2). From d 15 to 24, birds in both groups had similar FI, but those fed the HS diet gained more weight ($P = 0.033$) and as a result had a better FCR ($P < 0.001$). From d 24 to 28, birds fed the LS diet consumed significantly more feed than those fed the HS; however, BWG was not different ($P > 0.1$). Consequently, LS group had poorer FCR ($P = 0.003$). Over the whole experimental period (d 10 to 28), there was no difference in BWG ($P > 0.05$) between treatments. Still, birds in the LS group consumed more feed ($P = 0.021$), and thus were less efficient in feed conversion ($P < 0.001$) compared to the HS group.

Ileal digestibility coefficients and amylase activity

The freeze-dried weight of ileal digesta was significantly higher in birds fed the LS diet (containing 16.26% sand), resulting in lower ileal DM digestibility compared to those

Table 2. Effect of varying ratios of starch to fat on the overall production performance of broilers.[1]

	10–15 days			15–24 days			24–28 days			10–28 days		
Diets	FI[3]	BWG[3]	FCR[3]	FI	BWG	FCR	FI	BWG	FCR	FI	BWG	FCR
HS[2]	392 ± 12.0	267 ± 8.3	1.476 ± 0.04	931 ± 15.2	729 ± 8.3	1.277 ± 0.01	610 ± 4.9	424 ± 5.8	1.440 ± 0.02	1893 ± 28.5	1419 ± 20.1	1.334 ± 0.00
LS[2]	411 ± 4.1	272 ± 3.8	1.516 ± 0.02	948 ± 12.0	696 ± 11.9	1.364 ± 0.01	651 ± 4.8	433 ± 4.5	1.503 ± 0.01	1968 ± 16.9	1400 ± 12.8	1.406 ± 0.01
P-value*	0.149	0.977	0.184	0.488	0.033	<0.001	<0.001	0.371	0.003	0.021	0.106	<0.001

[1]Values are means ± SEM, n = 12 replicate pens of 80 birds each.
[2]HS and LS: high and low starch to fat ratio.
[3]FI: Feed intake (g/bird); BWG: Body weight gain (g/bird); FCR: Feed conversion ratio: FI/BWG.
* Differences between means are considered significant at $P < 0.05$.

Table 3. Effect of varying ratios of starch: fat on amylase activity (Unit/g jejunal chyme), nutrient concentration in ileal digesta[1] and ileal digestibility of nutrients[1] and energy.

			Freeze-dried ileal digesta		Ileal digestibility coefficients		
Age	Diets	Amylase activity[3]	Starch (g/kg)	Fat (g/kg)	Starch	Fat	Energy[3]
16 days	**HS**[2]	75.9 ± 10.7	80.3 ± 1.38	22.2 ± 0.12	0.950 ± 0.01	0.575 ± 0.03	-
	LS[2]	50.7 ± 10.6	58.1 ± 1.33	56.1 ± 0.50	0.893 ± 0.03	0.758 ± 0.02	-
	P-value*	0.1112	0.0665	< 0.001	0.0832	< 0.001	-
29 days	**HS**	74.3 ± 11.1	42.3 ± 0.46	18.0 ± 0.10	0.978 ± 0.00	0.690 ± 0.01	0.766 ± 0.01
	LS	51.1 ± 7.8	29.2 ± 0.45	29.0 ± 0.20	0.950 ± 0.01	0.878 ± 0.01	0.747 ± 0.01
	P-Value*	0.0831	0.0148	< 0.001	0.0094	< 0.001	0.1076

[1]Values are means ± SEM; n = 12 replicate pens of 2 birds each
[2]HS and LS: high and low starch to fat ratio
[3] n = 12 replicate pens of 1 bird each
* Differences between means were considered significant at P < 0.05

Table 4. Relationships between age and the apparent ileal digestibility coefficients [1] of starch and fat in broilers.

	HS diet [2]		LS diet [2]	
Age	Starch digestibility	Fat digestibility	Starch digestibility	Fat digestibility
16 d	0.950 ± 0.009	0.575 ± 0.028	0.893 ± 0.027	0.758 ± 0.019
29 d	0.978 ± 0.002	0.690 ± 0.015	0.950 ± 0.076	0.878 ± 0.007
P-values*	0.007	0.002	0.145	< 0.001

[1]Values are means ± SEM; n = 12 replicate pens of 2 birds each
[2]HS and LS: high and low starch to fat ratio
* Differences between means were considered significant at P < 0.05

fed the HS diet (data not shown). As shown in Table 3, starch content in the ileum varied between 29 and 80 g/kg digesta, and was significantly influenced by diet composition. Starch digestibility tended to be higher on d 16 (P = 0.083), and was higher (P = 0.009) on d 29 in birds fed the HS diet. The apparent fat digestibility was significantly higher in an LS diet group at both ages, while the apparent energy digestibility was not different (P > 0.05) between the treatments. On d 29, there was a tendency (P = 0.083) for higher amylase activity (by 45%) in the jejunum of birds fed the HS diet compared to the LS diet. Whereas the digestibility of fat was improved with bird age in both diet groups, starch digestibility was increased with age in the HS group only (Table 4).

Discussion

The current experiment demonstrated the large flexibility of broilers in terms of capacity to thrive on diets containing large variations in the ratios of starch to fat and high level of sand as an inert filler. Compared to the LS diet, feeding the HS diet was expected to cause a reduction in starch digestibility, which, in turn, might impair production performance and intestinal health. However, the HS diet was associated with improved, rather than impaired, starch digestibility and production performance.

Although ileal starch levels were higher in HS birds than LS birds, none of the examined bird groups were recorded with average concentrations higher than 80 g/kg ileal DM. Previous studies (Svihus and Hetland 2001; Svihus et al. 2010) which examined the association between dietary manipulations (pellets *vs*. mash and ground wheat *vs*. whole wheat) and ileal concentration of starch indicated that treatments associated with low (0.79–0.82) starch digestibility coefficients had mean ileal starch concentrations ranging from 222 to 250 g/kg ileal dry matter, whereas treatments associated with high (0.95) starch digestibility had starch concentrations ranging from 88 to 101 g/kg. These experiments were conducted with dietary starch levels ranging from 42% to 52%, as compared to 45% starch in our HS diet. These data indicate that ileal starch contents in the present experiment were similar to or lower than those found in bird groups with satisfactory starch digestibility in previous studies. Based on these data it was concluded that the intake of starch did not imply an overload in the gut in any experimental group in the current study. Poor starch digestibility in wheat diets has been attributed to several different factors, including the soluble fibre-fraction in wheat (Annison 1993), wheat hardness (Carré et al. 2002), resistant cell wall material (Meng et al. 2005), and a lower starch gelatinisation degree (Zimonja and Svihus 2009). The wheat in the current experiment was finely ground, and the diets were supplied with fibre-degrading enzymes to eliminate any potential effect of the cell wall or insoluble fibre fraction on nutrient encapsulation and digesta viscosity.

The surprisingly higher starch digestibility obtained with feeding the HS diet and the unanticipated lower starch digestibility associated with feeding the LS diet may be explained by unintended confounding factors, not least the observed higher extent of gelatinisation (by 7.3-fold) in the HS diet compared with the LS diet. A high degree of gelatinisation increases the susceptibility of starch to enzymatic hydrolysis (Mollah et al. 1983; Holm et al. 1988; Ankrah et al. 1999; Zimonja and Svihus 2009). The 14% difference in hot pellet temperature between the diets clearly indicated that, like soy oil (Cutlip et al. 2008), rapeseed oil in the LS diet had a lubricating effect, and as a result, decreased friction in the pellet die, which was the only source of heat at that point. This was supported by the pellet mill throughput and energy consumption data. In contrast, the very low oil content in the HS diet led to increased friction in the die, i.e., higher pellet temperature, and consequently higher degree of starch gelatinisation (Thomas et al. 1998). It is important to note that, although the LS diet resulted in lower starch digestibility, the average concentration of undigested starch in ileal contents was not higher than 58 g/kg, and tended to be lower (on d 16) or was significantly lower (on d 29) than ileal starch levels in the HS group.

It has been shown that starch gelatinisation can be modified, delayed or inhibited by the presence of lipids (Larsson 1980; Eliasson et al. 1981; Lund and Lorenz 1984). Lipids are known to form inclusion compounds with amylose (Putseys et al. 2010; López et al. 2012) during processing or in the intestine (Holm et al. 1983) which potentially, hinders starch digestion. Due to its hydrophobic nature, fat may interfere with the hydration of feed components, for example, by coating starch granules and limiting steam penetration (Zimonja et al. 2007), thus repressing swelling and

solubilisation (Eliasson et al. 1981; Svihus et al. 2005) and reducing the rate of starch hydrolysis (Tufvesson et al. 2001). Therefore, fat digestibility, or, in other words, the amount of undigested fat remaining in the intestine may have an impact on starch digestion. In fact, fat digestibility improved with age and was significantly higher with a low ratio of starch to fat. Although not evaluated, this may be due to an increase in fatty acid-binding protein activity, lipase activity and bile salt secretion (Krogdahl 1985; Krogdahl and Sell 1989). Compared to d 16, birds killed on d 29 in both dietary-groups had higher fat digestibility, i.e., less fat was present to complex with starch (Crowe et al. 2000). This would make the starch more available for amylase digestion especially since amylase activity was similar at both ages. Despite this, starch digestibility did not improve significantly with age in birds fed the LS diet. This suggested that the low ratio of starch to fat (high dietary level of fat) was not optimal for efficient starch utilisation under the experimental conditions applied. Several researchers (Nitsan et al. 1997; Veldkamp et al. 2017b) reported a decrease in starch digestibility with low compared to the high ratio of starch to fat in the diet.

Another plausible cause for the high starch digestibility associated with the HS diet was the use of the isolated wheat starch. This source was added to increase starch content in the diet, which was hypothesised to cause high concentrations of starch in the lower intestinal tract. Evidently, isolated wheat starch was not challenging enough for the birds, suggesting a fast rate of degradation in the upper intestinal tract. Compared to wheat, isolated wheat-starch was found to be hydrolysed more readily in vitro (Wiseman et al. 2000) and was completely digestible *in vivo* (Rogel et al. 1987), independent of the wheat characteristics (high or low AME).

Amylase results showed a trend characterised by an increase or decrease in activity depending on the amount of substrate in the digesta, as demonstrated previously (Karasov and Hume 1997). This physiological adaptation (Murugesan et al. 2014) may, at least partly, explain the high capacity of the birds to digest high levels of starch in the diet.

The lower apparent fat digestibility of the HS diet may have been attributed to the low content of dietary fat (14.2 g/kg) and a relatively higher contribution of endogenous losses, such as bile acid esters, cholesterol or structural lipids from desquamated cells (Jørgensen et al. 1993). It may be that broilers have a large capacity to utilise fat; however, due to the very low-fat content in the HS diet, fat digestibility from this group may have been unreliable.

The two diets differed significantly with regard to overall feed conversion ratio, but not with regard to body weight gain and ileal energy digestibility. A possible explanation could be that the amount of metabolisable energy was slightly different between the diets, although this was not intended. Both diets were formulated to be isoenergetic and isonitrogenous, assuming an AMEn value of 37.7 MJ/kg or 8843 kcal/kg for the rapeseed oil (Sauvant et al. 2004). However, the energetic value of rapeseed oil has been reported to vary considerably (8000–8500 kcal/kg rapeseed oil) (Scheele et al. 1997), and thus, the value used in the current trial calculations may have overestimated the true amount of metabolisable energy. Another factor which may have accounted in part for the better feed conversion in birds fed the HS diet was the decreased ingredient segregation (higher gelatinisation) and the resulting reduction of energy expenditure from feed intake. The potential role of an *Eimeria spp*. infection as an additional factor that may have influenced the production performance results is discussed in the accompanying paper (Granstad et al. in press).

The use of isolated wheat starch and the unintentionally higher extent of starch gelatinisation may have contributed to the high starch digestibility in birds given the HS diet. Thus, the hypothesis that high ratio of starch to fat in a pelleted diet may impair starch digestibility and production performance in *Eimeria*-challenged broiler chickens was not verified. Further work is required to clarify this research question, taking into consideration the physical form of starch source and the potentially confounding role of feed processing on starch availability.

Disclosure statement

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Paper III

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Varying starch to fat ratios in pelleted diets: II. Effects on intestinal histomorphometry, *Clostridium perfringens* and short-chain fatty acids in *Eimeria*-challenged broiler chickens

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Varying starch to fat ratios in pelleted diets: II. Effects on intestinal histomorphometry, *Clostridium perfringens* and short-chain fatty acids in *Eimeria*-challenged broiler chickens

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ABSTRACT

1. The hypothesis behind the study was that a high dietary starch level (HS) would lead to impaired gut health compared to a low-starch diet (LS) in *Eimeria*-challenged broilers. The effects of two diets with different starch to fat ratios on intestinal histomorphometry, *Clostridium perfringens* counts and toxin profile, necrotic enteritis prevalence and abundance of short-chain fatty acids (SCFAs) were examined.
2. A total of 1,920 one-day-old Ross 308 broiler chickens were fed one of two isocaloric diets formulated either with high (32:1) or low (2:1) starch to fat ratios from d 10 to 29 of age. Each treatment group had 12 pen replicates containing 80 broilers each. On d 17, the chickens were challenged with *Eimeria* vaccine strains. Samples were collected on d 16, 21–23 and 29.
3. Whereas villus length increased gradually throughout the study in the HS group, a peak level was reached on d 21–23 in the LS group. On d 29, the HS group had significantly longer villi than the LS group.
4. Caecal SCFA concentrations were higher in the HS group compared to the LS group on d 16. In both groups, the SCFA level peaked on d 21–23, with the most pronounced increase seen in the LS group.
5. The *C. perfringens netB:cpa* ratio increased from d 16 to 29 in the HS group. *C. perfringens* counts and necrotic enteritis prevalence were similar between the two groups.
6. Diet affected the dynamics of small intestinal villus length and caecal SCFA abundance. These findings suggest that structural remodelling of the small intestine is an adaptation to different dietary starch levels, and that caecal SCFA abundance is associated with the availability of substrate for the microbiota in the posterior intestinal segments. Chickens adapted to higher levels of dietary starch might be more robust against *Eimeria* infections due to increased mucosal surface area. Studies with other dietary starch sources are required to clarify the impact of dietary starch levels on intestinal health in *Eimeria*–challenged broilers.

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Introduction

Energy in broiler diets is mainly provided by carbohydrate and fat sources. Starch is, due to availability and cost-efficiency, often considered the primary source of energy, and is typically provided from cereals which, quantitatively, are the most important components in modern poultry diets (Svihus 2014; Zaefarian et al. 2015). Hence, the proportion of carbohydrates in the diet is much higher for modern broilers than for their wild ancestors. It has been estimated that insects can comprise more than 50% of the diet in wild jungle fowl chicks (Klasing 2005), and fat is, therefore, a more important energy-providing nutrient in an omnivore versus a granivore diet (Barker et al. 1998).

Intensive artificial selection through breeding for efficiency and growth have resulted in modern broiler breeds with a larger appetite and increased voluntary feed intake per day (Tallentire et al. 2016). The ability of broilers to digest nutrients does not necessarily always match their feed intake, and excessive undigested nutrients may reach the lower intestines (Svihus 2011). Substrate preferences differ between microorganisms, and the intestinal microbiota composition is largely determined by nutrient availability and chemical composition of the digesta (Apajalahti et al. 2004). As an example, the bacterium *Clostridium perfringens* (associated with necrotic enteritis in broilers) produces enzymes necessary to break down and utilise starch and sugar (Groves and Grounlund 1969; Shih and Labbe 1996), and the risk of necrotic enteritis has been linked to specific dietary carbohydrate sources (Annett et al. 2002; Kaldhusdal and Hofshagen 1992; Kaldhusdal and Skjerve 1996; Riddell and Kong 1992). Dietary fat sources may influence the growth of *C. perfringens*, and it has been shown that animal fat can increase *C. perfringens* counts compared to vegetable oil (Knarreborg et al. 2002). Consequently, it is possible to influence the growth of potentially pathogenic bacteria through dietary manipulation.

Furthermore, it is well established that coccidiosis caused by *Eimeria spp*. impairs intestinal health in its own right, and is an important predisposing factor for intestinal *C. perfringens* proliferation and the development of necrotic enteritis in broilers (Hermans and Morgan 2007). *Eimeria spp*. infections are common in commercial flocks, and display a dynamic pattern characterised by low infection levels in the youngest chicks and a peak infection level at an age interval that varies with *Eimeria spp*., farming system and health management. Short-chain fatty acids (SCFAs) are produced in the posterior intestine mainly

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from microbial fermentation of carbohydrates which escape absorption in the small intestine (Morrison and Preston 2016). There are numerous effects associated with SCFAs, and some of them are involved in host energy metabolism, energy supply, intestinal function and epithelial cell morphology (den Besten et al. 2013; Scheppach 1994). The amount and type of fermentable substrates reaching the lower intestinal segments affects the composition of the intestinal microbiota and, hence, the SCFA concentration and profile (Cummings and Macfarlane 1991). Undigested dietary carbohydrates primarily lead to the formation of volatile fatty acids; acetate, propionate and butyrate (Wong et al. 2006). A specific diet that leads to increased production of SCFAs lowers the pH in the intestine, which could create a favourable environment for some microbes and disadvantageous conditions for others (den Besten et al. 2013).

The ability to digest and absorb carbohydrates is believed to be highly adaptable in poultry (Murugesan et al. 2014; Suvarna et al. 2005), and the digestive capacity of the intestine can be increased through the expansion of surface area which occurs with lengthening of villi (Moran 1985). The turnover rate of small intestinal epithelium in chickens allows for rapid adjustments of crypts and villi, ensuring that chickens can adapt quickly to altered nutrient availability (Imondi and Bird 1966).

The following study was based on the hypothesis that a HS diet would result in excessive amounts of undigested starch reaching the distal part of ileum and caecum, which could affect the count and toxin gene profile of *C. perfringens* and the prevalence of necrotic enteritis. Based on the considerations above, it was expected that the different macronutrient contents in the HS and the LS diet would influence small intestinal histomorphometry and production of SCFAs. The objective of this study was to investigate the effects of two diets with different starch to fat ratios on intestinal histomorphometry, *C. perfringens* counts and toxin profile, occurrence of necrotic enteritis and abundance of short-chain fatty acids (SCFAs) in *Eimeria*-challenged broiler chickens. Effects on nutrient digestibility and production performance have been discussed in an accompanying paper (Itani et al. in press).

Materials and methods

Animals and housing

The experiment was approved by the national animal research authority (Norwegian Food Safety Authority, approval ID 8824), and performed in accordance with national and international guidelines for the care and use of experimental animals. The housing, management and environment are described in detail in an accompanying paper (Itani et al. in press). Briefly, 1,920, one-day-old Ross 308 broiler chickens were obtained from a commercial hatchery (Nortura Samvirkekylling, Våler, Hedmark, Norway) and housed in floor pens on new wood shavings in a climate-controlled poultry research facility (Scandinavian Poultry Research, Våler, Hedmark, Norway). Water and feed were provided *ad libitum*.

Diets & experimental design

From d 0 to d 9, all chickens were fed a common commercial starter diet containing 366 g/kg starch (mostly derived from wheat) and 61 g/kg fat (mostly derived from soybean oil and animal fats), providing a starch to fat ratio of 6:1. On d 10, the chickens were randomly divided into two treatment groups, both comprising 12 pens with 80 chickens each with a balanced male to female distribution per pen. During the experimental period from day 10 to day 29, chickens in the two treatment groups were fed either a high starch to fat ratio (HS; ratio of 32:1) or a low starch to fat ratio (LS; ratio of 2:1) diet. The diets were formulated to be isocaloric by replacing wheat starch in the HS diet by rapeseed oil and silica sand in the LS diet. Both diets were pelleted and formulated to meet or exceed the Ross 308 nutrition specifications (Aviagen 2014). All diets were free from in-feed antimicrobials, including antibacterial growth promoters and anticoccidials. More details on diet composition and feed processing are described in the accompanying paper (Itani et al. in press).

On d 17, chickens in both treatment groups were challenged orally with a 10-fold dose of the vaccine Paracox-5 vet. (MSD Animal Health, Boxmeer, the Netherlands) containing live, sporulated oocysts from five attenuated strains of *Eimeria spp*. (one precocious line each of *Eimeria acervulina* [approximately 5750 oocysts per broiler], *Eimeria mitis* [approximately 11,500 oocysts], and *Eimeria tenella* [approximately 5750 oocysts], and two precocious lines of *Eimeria maxima* [approximately 3450 oocysts]) administered through the drinking water. On d 16, 21–23 and 29, 12 chickens per treatment group (one chicken from each replicate pen) were randomly selected and euthanised by a cranial blow immediately followed by cervical dislocation before necropsy and sample collection.

Morphometric analysis

Unopened intestinal segments of approximately 1 cm taken from the transition between duodenum and jejunum at the end of the duodenal loop were collected during necropsy on d 16, 21–23 and 29. The samples were fixed in 10% neutral-buffered formalin solution. Formalin-fixed intestinal tissue samples were embedded in paraffin and sectioned at 5 µm. Standard protocols for haematoxylin and eosin staining of histological paraffin sections were followed. Villus length and crypt depth were measured using a microscope (Nikon Eclipse 80i, Nikon Instruments Europe B.V., Amsterdam, the Netherlands) fitted with a digital camera (Nikon DS-Ri1, Nikon Instruments Europe B.V.) using the image software NIS-Elements D v4.40 (Nikon Instruments Europe B.V.). Villus length was measured from the tip of the villus to the crypt-villus junction. Crypt depth was measured from the base to the crypt-villus junction. The mean of ~10 measured villi and crypts per chicken was calculated. Only clearly defined and fully finger-shaped villi were included and histological sections of suboptimal quality not suitable for morphometric analysis were excluded. Mean villus length, crypt depth and villus:crypt ratio in each treatment group were calculated.

Clostridium perfringens *quantification and toxin gene analysis*

On d 21, 22 and 23 (d 4, 5 and 6 after *Eimeria* challenge, respectively) caecal samples (1.0–2.0 g) were collected in sterile stomacher bags and immediately subjected to cultivation in order to quantify *C. perfringens*. Briefly, caecal samples were diluted 1:100 in peptone saline water (0.1% peptone, Difco Laboratories Inc., Detroit, MI, USA, and 0.85% NaCl) and homogenised for 30 seconds (Bagmixer

400 CC, Interscience, Saint Nom, France). Serial dilutions were made with non-buffered peptone water until a dilution of 10^{-6} was reached. Aliquots of 100 µl from the dilutions 10^{-2}, 10^{-4} and 10^{-6} were plated onto sheep blood agar plates and incubated anaerobically at 37°C for 24 hours (Genbox anaer, Biomérieux, Marcy-l'Étoile, France). Single colonies with double haemolysis were counted, and colony-forming units per gram (cfu/g) of caecal content was calculated. Typical colonies were selected for pure cultivation and confirmed as *C. perfringens* by a matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Bruker Corp., Billerica, MA, USA).

Caecal contents were collected in 1.5 ml Eppendorf tubes and immediately put on dry ice during necropsy on d 16, 21–23 and 29. Samples were stored at −80°C until further processing. DNA was extracted from 200 mg of caecal content using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with some modifications. After adding 1.4 ml of ASL lysis buffer, the samples were homogenised on a vortexer, followed by heat treatment at 70°C for 5 min. The total suspension from each sample was transferred into Precellys 2.0 ml homogenisation tubes prefilled with 0.1 mm ceramic beads (Bertin Technologies, Montigny-le-Bretonneux, France). Mechanical lysis was done with a Precellys 24 homogeniser (Bertin Technologies) for 2 × 30 seconds at 6800 rpm. Samples were centrifuged for 1 min at 14.000 rpm in order to pellet both the beads and large particles. The supernatant was transferred into new 2 ml tubes and heated at 70°C for 5 min. One InhibitEX tablet was added to each sample, and, for the rest of the procedure, the protocol provided by the manufacturer was followed. DNA yields were measured with the Qubit double-stranded DNA Broad-Range Assay Kit on a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The purity of the DNA extracts was determined by measuring the ratios of absorbance at 260/280 and 260/230 using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). DNA was stored at −20°C until use.

The relative abundances of the *C. perfringens* pathogenicity-associated necrotic enteritis toxin B-like gene (*netB)* and the omnipresent alpha toxin gene (*cpa)* were analysed using a multiplex real-time qPCR in order to determine the ratio of presumptively pathogenic to total *C. perfringens* (*netB:cpa* ratio) in intestinal samples. The qPCR assay used primers and probes as described previously (Albini et al. 2008; Schlegel et al. 2012) with some modifications (Table 1). *C. perfringens* strain 56 (CP56) was used as positive control, and nuclease-free water was used as negative control. The CP56 was originally isolated from the gut of a broiler chicken with severe necrotic enteritis lesions, and the strain has previously been classified as a *netB* toxin positive strain (Gholamiandekhordi et al. 2006). The *cpa* gene served as an internal positive control for each PCR reaction due to its presence on the chromosome of all *C. perfringens* isolates (Songer 1996). Each reaction was performed in duplicate in a 25 µl total reaction mixture containing 12.5 µl Brilliant III Ultra-Fast qPCR Master Mix (Agilent Technologies, Santa Clara, California, US), 20 µM of primers and probes, and 3 µl of the DNA sample. A Bio-Rad CFX-96 real-time PCR instrument (Bio-Rad Laboratories Inc., Hercules, California, US) was used. The qPCR conditions were 1 cycle at 50°C for 10 min and 1 cycle at 95°C for 5 min, followed by 48 cycles at 95°C for 30 s and 60°C for 1 min. Data analysis was performed using Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories Inc.) and Microsoft Office Excel 2016 (Microsoft, Redmond, Washington, US). The standard curve was based on data from serial 10-fold dilutions (10^{0}–10^{-4}) of DNA from CP56. Amplification efficiency for both PCRs were calculated from these titrations to confirm that it was within the range of 90–110%. The slope and shape of amplification curves were inspected and quantification cycle (Cq) thresholds above 40 were excluded from the analysis. The threshold lines were set at the same level for both PCRs, at a point above the background fluorescence and in the beginning of the exponential phase. The amplification signals from the target genes in the undiluted positive control CP56 overlapped at this point, indicating that the ratio of *netB* to *cpa*, based on Cq values for this positive control, was 1:1. The percentage of *netB*-positive samples was calculated based on the presence or absence of the *netB* gene signal. The relative abundances of the *cpa* and the *netB* genes were determined from the standard curve, and the *netB:cpa* ratios in caecal samples were calculated by dividing the relative *netB* abundance by the relative *cpa* abundance.

Short chain fatty acids

Caecal samples were collected in Eppendorf tubes and immediately put on dry ice during necropsy on d 16, 21–23 and 29. Samples were stored at −80°C until further analysis. The SCFA concentrations in the caecal samples were determined according to a previously described method (De Weirdt et al. 2010). In short, SCFAs were extracted using diethyl ether, and 2-methylhexanoic acid (99%) was added to each sample as internal standard. The extracts were analysed using a GC-2014 gas chromatograph (Shimadzu, BB 's-Hertogenbosch, the Netherlands), equipped with a capillary fatty-acid free EC-1000 EconoCap column (Alltech, Laarne, Belgium).

Necrotic enteritis

The small intestine of 12 chickens (one per replicate pen) per treatment were opened longitudinally and inspected for pathological changes indicating necrotic enteritis on d 16, 21, 22, 23 and 29. A modified necrotic enteritis scoring system was used (Lovland et al. 2004). If no macroscopic mucosal ulcers, depressions or pseudomembranes were present, the chickens were classified as 'necrotic enteritis

Table 1. Primers and probes used for qPCR analysis.

Target	Description	Nucleic acid sequences 5′ to 3′ with probe dyes (Tm)	Product length (bp)	Reference
cpa	Forward primer	AAG AAC TAG TAG CTT ACA TAT CAA CTA GTG GTG (57.3°C)	124	(Albini et al. 2008)
	Reverse primer	TTT CCT GGG TTG TCC ATT TCC (55.4°C)		
	Probe	HEX-TTG GAA TCA -ZEN- AAA CAA AGG ATG GAA AAA CTC AAG-IBFQ (58.4°C)		
netB	Forward primer	GGC GGT AAT ATA TCT GTT GAA GG (53.1°C)	168	(Schlegel et al. 2012)
	Reverse primer	ACC GTC CTT AGT CTC AAC (51.0°C)		
	Probe	FAM-ACT GCT GGT -ZEN- GCT GGA ATA AAT GCT TCA-IBFQ (60.8°C)		

negative'. Identification of minimum one mucosal ulcer, depression or pseudomembrane resulted in classification as a 'necrotic enteritis positive' chicken.

Gizzard scores

Gizzards from 12 chickens per treatment (one per pen replicate) on d 16, 21, 22, 23 and 29 were opened, emptied and carefully washed in order to inspect the gizzard linings. A score for each gizzard was given on the basis of score 0 for no visible erosions (negative), 1 for definite erosions with restricted or focal distribution (mild), 2 for multiple erosions with widespread distribution but more than 50% normal mucosa remaining (moderate) and 3 for erosions covering more than 50% of the gizzard surface area (severe).

Statistical analysis

Samples were collected from one chicken from each replicate pen (12 chickens per treatment group) on each sampling occasion. Initially, data from all variables were checked for normality and homogeneity of variance using Shapiro-Wilk test and Variance-comparison test, respectively. If the criteria for using parametric testing were fulfilled (normal distribution and homogeneity of variance), an independent two-sample t-test was used. If the assumptions of normality and/or homogeneity of variance were violated, the non-parametric Mann-Whitney U test (Wilcoxon rank-sum test) was used. For categorical data, Fisher's exact test was used. Choice of statistical tests for each outcome variable are given in the results sections and/or in tables and figures. Differences between means were considered significant at $P < 0.05$. Statistical analyses were performed using Stata version 14.2 (StataCorp LLC, College Station, TX, USA) and graphics were made using R version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Morphometric analysis

There was no significant difference in mean villus length, crypt depth or villus:crypt ratio in the duodenojejunal junction between the two groups on d 16 and 21–23 (Table 2, t-test). On d 29, chickens fed the HS diet had longer villi compared to chickens fed the LS diet ($P < 0.001$). There was no difference in mean crypt depth on d 29. As a result of the difference in villus length, the mean villus:crypt ratio on d 29 was higher in chickens fed the HS diet compared to chickens fed the LS diet ($P = 0.037$).

The length of villi in the HS group increased gradually from d 16 to day 29 (Table 2). This increase was most pronounced between d 16 and d 21–23 (25% increase, $P = 0.001$, t-test). In the LS group, villus length increased strongly from d 16 to d 21–23 (39% increase, $P < 0.001$, t-test), but decreased in the period from d 21–23 to 29 (13% decrease, $P = 0.045$, t-test).

Table 2. Effect of dietary starch to fat ratios on intestinal histomorphometry on days 16, 21–23 and 29.[1]

		Diet[2]		
Age	Variable	HS	LS	P-value[3]
16 days	Villus length (µm)	1255 ± 33	1187 ± 30	0.154
	Crypt depth (µm)	175 ± 5	179 ± 4	0.493
	Villus:crypt ratio	7.23 ± 0.2	6.67 ± 0.3	0.153
21–23 days	Villus length (µm)	1570 ± 78	1650 ± 83	0.497
	Crypt depth (µm)	175 ± 7	192 ± 7	0.111
	Villus:crypt ratio	9.09 ± 0.6	8.74 ± 0.6	0.669
29 days	Villus length (µm)	1689 ± 48	1440 ± 33	<0.001
	Crypt depth (µm)	174 ± 5	176 ± 8	0.875
	Villus:crypt ratio	9.83 ± 0.5	8.32 ± 0.4	0.037

[1]Values are means ± SEM.
[2]HS: high starch to fat ratio diet; LS: low starch to fat ratio diet.
[3]Independent two-sample t-test.

Clostridium perfringens quantification and toxin gene analysis

There was no significant difference in caecal *C. perfringens* counts (cfu/g) between the two treatment groups between d 21–23 ($P = 0.098$, Mann-WhitneyU test) (Figure 1).

The qPCR analysis revealed that all caecal samples from both treatment groups were *cpa*-positive. The frequency of samples with presence of the *netB* gene was calculated (Table 3). From d 16 to d 29, the overall percentage of *netB*-positive caecal samples in both treatment groups increased from 79% to 100% ($P = 0.05$, Fisher's exact test). There was no statistically significant effect of diet on *netB* prevalence at any time point or in the overall experimental period.

The *netB:cpa* ratio was similar (Figure 2) in the two treatment groups on d 16 ($P = 0.354$), d 21–23 ($P = 0.624$) and d 29 ($P = 0.299$). In the HS group, the *netB:cpa* ratio increased from d 16 to 29 ($P = 0.018$, Mann-Whitney U test), indicating that the relative abundance of *netB* genes present in caecal samples from chickens fed the HS diet increased to a greater extent than the relative abundance of *cpa* genes in this period. The HS group on d 29 was the only subgroup with mean and median *netB:cpa* ratio above 1.0, indicating that the relative abundance of *netB* was higher than the relative abundance of *cpa* in this group at this time point.

Short chain fatty acids

On d 16, caecal samples from chickens fed the HS diet had higher levels of acetic acid ($P = 0.016$) and propionic acid ($P = 0.006$) compared to caecal samples from chickens fed the LS diet (Table 4). This resulted in higher total SCFA concentration ($P = 0.016$) in the caecum from chickens fed HS diet at this time point.

The dynamic development of total caecal SCFA concentration was similar in the two diet groups, with a maximum level reached on d 21–23. The increase from d 16 to d 21–23 was significant in the LS group ($P < 0.001$, t-test).

Necrotic enteritis

The overall prevalence of necrotic enteritis on d 16 was 8.3% (2/24 chickens). In the period following *Eimeria* challenge (d 21–23) the prevalence was 16.7% (6/36 chickens) in the HS group and 13.9% (5/36 chickens) in the LS group. This difference in necrotic enteritis prevalence was non-significant ($P > 0.05$, Fisher's exact test). On d 29, no mucosal ulcers, depressions or pseudomembranes indicating necrotic enteritis were detected in the small intestine of any of the chickens examined.

Gizzard scores

On d 16, the mean gizzard score was 2.0 and 1.75 in the HS and LS group, respectively ($P = 0.508$, t-test). During the days

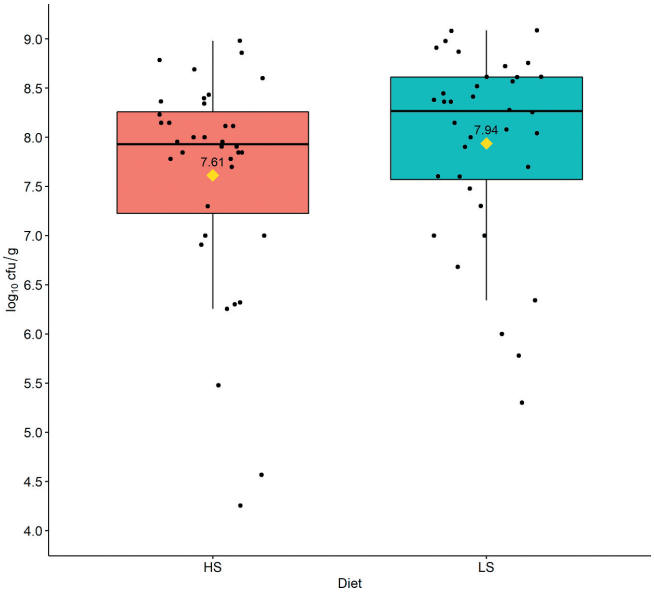

Figure 1. Effect of dietary starch to fat ratios on caecal *Clostridium perfringens* counts (log_{10} cfu/g) during days 21–23. Boxplots with medians indicated by horizontal lines and means indicated by diamonds. Based on samples from 36 chickens per diet. HS: high starch to fat ratio diet; LS: low starch to fat ratio diet. cfu/g = colony forming units per gram.

Table 3. The percentage of *netB*-positive and *netB*-negative caecal samples.[1]

Age	Diet	*netB*-negative		*netB*-positive	
16 days	HS	25%	21%	75%	79%
	LS	17%		83%	
21–23 days	HS	8%	8%	92%	92%
	LS	8%		92%	
29 days	HS	0%	0%	100%	100%
	LS	0%		100%	
Total			10%		90%

[1]Based on samples from 12 chickens per diet per time point.
HS: high starch to fat ratio diet; LS: low starch to fat ratio diet.

following *Eimeria* challenge (d 21, 22 and 23), the overall median gizzard score was 3 in both treatment groups (P = 0.489, Mann-Whitney U test). At the end of the experiment on d 29, the mean gizzard score was 2.7 in chickens fed the HS diet and 2.8 in chickens fed the LS diet (P = 0.368, t-test). There was no effect of diet on gizzard scores in the experiment.

Discussion

The use of intestinal *C. perfringens* counts as an indicator of gastrointestinal health in this study is based on this bacterium's association with gizzard erosions (Novoa-Garrido et al. 2006), necrotic enteritis (Kaldhusdal and Hofshagen 1992) and growth depression (Stutz and Lawton 1984) in broiler chickens. *C. perfringens* produces amylases and has the capacity to break down and utilise starch (Shih and Labbe 1996). Any undigested starch reaching lower gut regions could thus encourage overgrowth of this opportunistic pathogen. In spite of the higher starch content in the HS diet, this diet did not lead to higher caecal *C. perfringens* counts than the LS diet at d 21–23. As shown in the accompanying paper, the concentration of starch in ileal digesta tended to be higher on d 16 (28% more starch, P = 0.067), and was clearly higher on d 29 (31% more starch, P = 0.015) in the HS group compared to the LS group (Itani et al. in press). These findings indicated that more undigested starch reached the lower intestinal regions in chickens fed the HS diet, but the difference in ileal substrate availability at these time points did not affect caecal *C. perfringens* counts significantly during the most critical time interval after *Eimeria spp*., challenge (d 21–23). This result was in agreement with the lack of difference in overall gizzard scores and necrotic enteritis frequency between chickens fed the two diets. Neither of the treatment groups in this study had very high levels of starch in the ileum on the day before *Eimeria spp*. challenge (80 and 58 g/kg in the HS and LS group, respectively) unlike chickens in another study (222 g/kg) which had poor starch digestibility (Svihus and Hetland 2001). The relatively low levels of starch, and limited substrate availability in the posterior gut regions, could explain the lack of difference in important gut health variables between the treatment groups in this study. The presence of other microbes outcompeting *C. perfringens* in their use of starch as fermentation substrate is an additional or alternative possible explanation for the lack of impact of diet on caecal *C. perfringens* counts and necrotic enteritis prevalence.

In chickens, *C. perfringens* type G (previously designated type A) has been reported as the predominant toxinotype causing necrotic enteritis (Rood et al. 2018, Van Immerseel et al. 2004). The alpha-toxin-encoding gene *cpa* is present on the chromosome of all types of *C. perfringens* strains (Petit et al. 1999), and was used as an indicator of total *C. perfringens* abundance in caecal samples in a quantitative PCR (qPCR) assay in this study. Another toxin, the pore-forming plasmid-encoded toxin designated NetB, is believed to be a key virulence factor associated with necrotic enteritis in chickens (Keyburn et al. 2008). The current trial showed a relatively high percentage of *netB*-positive caecal samples on d 16 prior to *Eimeria spp*. challenge in (Table 3). In a previous study, *netB*-positive *C. perfringens* strains were present in a commercial broiler

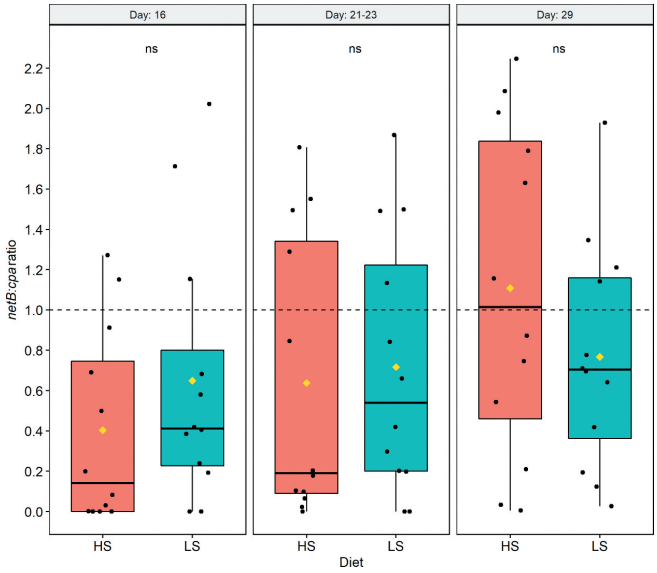

Figure 2. Effect of dietary starch to fat ratios on the *Clostridium perfringens netB:cpa* ratio in caecal samples on days 16, 21–23 and 29. Boxplots with medians indicated by horizontal lines and means indicated by diamonds. Based on samples from 12 chickens per diet per time point. Statistical analysis carried out by Mann-Whitney U test. HS: high starch:fat ratio diet; LS: low starch:fat ratio diet; ns = not significant.

Table 4. Effect of dietary starch to fat ratios on short chain fatty acids (SCFAs; μmol/g) in caecum.[1]

	Day 16			Day 21–23			Day 29		
	HS	LS	P-value	HS	LS	P-value	HS	LS	P-value
Acetic acid	58.24 ± 4.17	43.43 ± 2.95	0.016	66.75 ± 2.76	67.28 ± 4.99	0.729	62.34 ± 3.80	55.79 ± 4.37	0.184
Propionic acid	3.29 ± 0.33	1.85 ± 0.35	0.006	4.32 ± 0.49	4.39 ± 0.70	0.624	4.33 ± 0.24	4.09 ± 0.47	0.356
Butyric acid	10.21 ± 0.85	9.57 ± 0.95	0.580	12.96 ± 1.27	13.72 ± 0.86	0.419	11.24 ± 1.37	11.67 ± 1.31	0.773
Total SCFAs	73.94 ± 4.99	55.96 ± 3.78	0.016	86.89 ± 4.01	88.23 ± 5.65	0.356	80.64 ± 5.19	73.59 ± 5.82	0.299

[1]Values are means (μmol/g) ± SEM. Statistical analysis carried out by Mann-Whitney U test.
HS: high starch to fat ratio diet; LS: low starch to fat ratio diet.

house prior to placement of one-day-old broilers (Engstrom et al. 2012). From this background, it seems reasonable to suggest that *netB*-positive *C. perfringens* strains could have been present in the environment from placement of the one-day-old broilers in the current study. The frequency of *netB*-positive caecal samples increased from 16 to 29 d of age. Although no necrotic enteritis was detected on d 29, all caecal samples were *netB*-positive at this age. A possible explanation was that *netB*-positive strains were transmitted from broilers with subclinical necrotic enteritis to healthy birds, and eventually colonised the intestine of all broilers independent of health status. The interpretation of the presence of the *netB* gene with regard to pathogenicity of *C. perfringens* can be complex, and previous studies showed that *netB*-positive *C. perfringens* strains alone are apparently not enough to cause disease without predisposing factors being present (Keyburn et al. 2010; Yang et al. 2019).

The significant increase in *netB:cpa* ratio from d 16 to day 29 in the HS group implied that the relative abundance of *netB* increased to a greater extent than the relative abundance of *cpa* in this period. Since a significant increase in *netB:cpa* ratio was not present in the LS group, it was possible that diet and starch level played a role in this development. As mentioned before, *C. perfringens* can utilise starch, which was available in larger amounts in ileal contents in the HS group compared to the LS group (Itani et al. in press). Thus, starch may have predisposed birds towards intestinal colonisation by *netB*-positive *C. perfringens* in this study. The *netB:cpa* ratio has been suggested as a faecal marker to monitor subclinical necrotic enteritis in broilers (Goossens et al. 2019). The current study was not designed to compare *netB:cpa* ratios in groups with and without necrotic enteritis, but the results demonstrated that the *netB:cpa* ratio was not directly associated with prevalence of necrotic enteritis at the time of sampling. It is, however, possible that this ratio can be useful as an indicator of previous necrotic enteritis occurrence in the examined flock. More work is required to determine the use of this marker.

In addition to age-related lengthening of intestinal villi in broilers (Alshamy et al. 2018), villus length and development are influenced by feed composition and nutrient availability (Moran 1985). Elongation of villi increases the absorptive surface of the intestine and augments the amount of brush border enzymes available to break down oligosaccharides originating from the activity of amylase on dietary starch in the anterior small intestine. Hence, increased villus length and consequently expansion of the mucosal surface area is considered an important mechanism in adaptation to higher starch levels in the diet (Moran 1985). The difference in villus length between the HS and LS group on d 29 supported this view.

The development of villus length was similar in chickens fed the LS and HS diets during the time interval between d 16 and d 21–23. This similarity might have been associated with

the *Eimeria spp*. challenge on d 17, considering that lengthening of villi as a compensatory mechanism following *Eimeria acervulina* infection has been described previously (Fernando and Mccraw 1973). However, the two diet groups showed clearly distinct developments in villus length from d 21–23 to d 29. The decrease in villus length in the LS group was in contrast to the continued increase of villus length in the HS group. Both groups were challenged with *Eimeria spp*. and samples were taken at the same age. The difference in villus length development between d 21–23 and d 29 was thus most likely related to the impact of nutrients and diet. Digestion of fat and fatty acids is believed to occur mainly in the jejunum and the upper ileum (Tancharoenrat et al. 2014), while the greatest part of starch digestion occurs in the duodenum and jejunum (Osman 1982; Riesenfeld et al. 1980). Intestinal adaptation to increased levels of fat in the diet, such as raised expression of fatty acid binding proteins in epithelial cells (Krogdahl 1985), is thus likely to primarily take place in more posterior regions of the small intestine than adaption to increased starch levels. The results from this study suggested that a diet containing a higher level of starch stimulates villus elongation in the duodenojejunal junction to a larger extent than a diet containing less starch and more vegetable fat.

The higher total caecal SCFA concentration in chickens fed the HS diet compared to chickens fed the LS diet on d 16 implied a larger number of SCFA-producing bacteria in the intestines of this group (den Besten et al. 2013). The dynamic development of total caecal SCFA concentration showed a similar trend in both the HS and the LS group, with an initial increase and a maximum level reached during d 21–23, followed by a moderate and non-significant decrease towards the end of the experiment (Table 4). The increase in total SCFA concentration from d 16 to d 21–23 was, on average, 18% in the HS group and 58% in the LS group, which suggested a more considerable increase in activity of SCFA-producing bacteria in the LS group. A possible explanation for this may be that the infection process following exposure of both groups to the same dose of *Eimeria spp*. on d 17 affected a smaller area of available digestive surface in the HS group than in the LS group, since the HS group (from d 10) adjusted to the higher level of dietary starch by the relative increase of intestinal surface area and villus length in the anterior small intestine (Moran 1985). A reduction in starch digestibility associated with *Eimeria spp*. challenge (Amerah and Ravindran 2015) could have affected the HS group to a lesser extent compared to the LS group, due to the larger remaining functional intestinal surface area. Consequently, more starch potentially reached posterior gut regions in the LS group in this period (data not shown), resulting in larger amounts of substrates accessible for SCFA-producing bacteria and a more pronounced increase in total SCFA concentration. This reasoning was supported by the fact that starch digestibility was improved from d 16 to 29 in the HS group but not in the LS group (data presented in the accompanying paper Itani et al. in press).

There was no clear-cut difference in necrotic enteritis prevalence, caecal *C. perfringens* counts or gizzard scores between chickens fed the two diets in this study. The hypothesis that a HS diet would lead to unfavourable effects on intestinal health in broiler chickens compared to a LS diet was not confirmed in this study. As discussed in the accompanying paper, feed processing and the physical form of starch sources were potentially confounding factors (Itani et al. in press). The unintentionally higher extent of starch gelatinisation and the use of isolated wheat starch in the HS diet could have contributed to the relatively small difference in ileal starch level between the two groups, in spite of the marked difference in dietary starch content (45% in HS and 23% in LS). Taking this into account, the current data cannot be used to reject the hypothesis that high levels of dietary starch may impair intestinal health in broiler chickens.

In conclusion, there was an impact of diet on the length of anterior small intestinal villi and abundance of SCFAs in the caecum. A possible explanation for the difference in villus morphology was that the two groups had to adapt to distinct dietary macro-nutrient ratios, which required different ways of extracting energy from the feed. Due to ongoing adaptation to higher levels of dietary starch through villus elongation and increased mucosal surface (Moran 1985), chickens fed the HS diet were conceivably less vulnerable to the loss of absorptive capacity caused by the *Eimeria spp*. challenge. Chickens fed the LS diet had to rapidly adjust to loss of absorptive function in a larger proportion of the duodenal-jejunal mucosal surface in the period following challenge, and apparently accelerated the lengthening of villi in order to maintain nutrient absorption capacity. This structural remodelling of the intestine was energy-demanding, and this cost was reflected in reduced weight gain during d 15 to 24 and poorer accumulated feed conversion in the LS group (Itani et al. in press). The reduction in absorptive capacity of chickens fed the LS diet was underpinned by the more pronounced increase in total caecal SCFA concentration from d 16 to d 21–23 in this group, which strongly suggested that more undigested starch reached lower gut regions and were available for bacterial fermentation in this period. Collectively, these results suggested that chickens which were adapted to a diet with a higher level of starch were better prepared to cope with intestinal damage caused by a mild to moderate *Eimeria spp*. challenge affecting the anterior small intestine, compared to chickens fed a diet with a lower level of starch. This hypothesis was supported by the fact that the LS group had clearly poorer production performance during d 15–24 (Itani et al. in press), an age interval that included peak oocyst excretion following *Eimeria spp* challenge. However, because the study was not designed to investigate the relationship between dietary starch level and *Eimeria* infections, this hypothesis needs to be tested experimentally.

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Disclosure statement

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