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Feed for defence

Can dietary supplements modulate broiler gut microbiota and guard against *Campylobacter*?

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Cover: Rowan Ranger chicks drinking water inoculated with *Lactiplantibacillus plantarum* 256 (photo: E. Eliasson)

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Abstract

The bacteria *Campylobacter jejuni* is the causative agent of a disease in humans called campylobacteriosis and is the most commonly reported zoonosis in the European Union, with the primary source of infection often traced to broiler products. While low overall *C. jejuni* prevalence is reported in conventional broiler production in northern Europe, heightened incidences persist in summer months, particularly in organic production. Supplementing broiler diets with dietary components rich in lactic acid bacteria or complex polysaccharides have been identified as promising measures to decrease *Campylobacter* presence in various production systems. This thesis investigated the effect of daily consumption of *Lactiplantibacillus plantarum* 256 (LP256)-supplemented water, silage inoculated with LP256 and haylage (a non-inoculated forage similar to silage) on *C. jejuni* colonisation and microbiota composition. The effects of supplementing broiler feed with algal extract on development of caecal microbiota and colonisation by *C. jejuni* were also examined. Daily intake of LP256 and algal extract diet supplementation proved ineffective as interventions against *C. jejuni* colonisation. The treatments did not alter broiler microbiota composition, although shifts occurred after *C. jejuni* challenge, suggesting that colonisation affected the gut microbiota. Adding LP256 to water improved broiler initial weight. Inclusion of forages in the diet of slower-growing hybrids did not compromise performance, but in fast-growing hybrids, the incorporation of haylage showed adverse effects. These findings improve understanding of microbial interactions in the gut of broiler chickens, but the observed changes need to be validated in future research.

Keywords: *Campylobacter jejuni*, feed supplement, *Lactiplantibacillus plantarum* 256, forage, algal extract, laminarin, broiler, caecal microbiota

Foder för försvar

Kan fodertillskott modulera broilers tarmmikrobiota och skydda mot *Campylobacter*?

Sammanfattning

Bakterien *Campylobacter* jejuni orsakar sjukdomen campylobacterios hos människor och är den mest rapporterade zoonosen inom den Europeiska unionen. Den primära infektionskällan kan ofta spåras till kycklingprodukter. Även om låg förekomst av *C. jejuni* rapporteras i konventionell kycklingproduktion i norra Europa, så ökar antalet drabbade flockar under sommarmånaderna, särskilt inom ekologisk produktion. Tillskott av fodermedel med högt innehåll av mjölksyrabakterier eller komplexa polysackarider har identifierats som lovande åtgärder för att minska incidensen av *Campylobacter* i olika produktionssystem. Denna avhandling undersökte hur dagligt intag av vatten med tillsats av *Lactiplantibacillus plantarum* 256 (LP256), ensilage inokulerat med LP256 och hösilage (ett icke-inokulerat foder liknande ensilage) påverkade etablering av *C. jejuni* och mikroflorans sammansättning i kycklingens blindtarmar. Tillsats av algextrakt i kycklingfoder och dess effekt på mikroflorans utveckling i blindtarmarna, samt etablering av *C. jejuni*, undersöktes också. Dagligt intag av LP256 och algextrakt visade sig vara ineffektiva mot etableringen av *C. jejuni*. Behandlingarna förändrade inte mikroflorans sammansättning heller, däremot observerades ett skifte efter avsiktlig infektion med *C. jejuni*, vilket tyder på att etableringen påverkade mikrofloran. Tillsats av LP256 i vattnet ledde till högre vikt i början av tillväxtperioden. Inkludering av grovfoder i foderstaten påverkade inte foderintag eller tillväxt hos långsamtväxande hybrider, medan det observerades en negativ inverkan av hösilage hos snabbväxande hybrider. Dessa resultat bidrar till en ökad kunskap om bakteriella interaktioner i kycklingens tarm, dock behövs mer forskning för att förstå de bakomliggande orsakerna.

Nyckelord: *Campylobacter jejuni*, fodertillskott, *Lactiplantibacillus plantarum* 256, grovfoder, algextrakt, laminarin, slaktkyckling, tarmflora

Preface

"The alchemists in their search for gold discovered many other things of greater value." —Arthur Schopenhauer

Dedication

To my beloved parents
Irena & Pavel

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Mogodiniyai Kasmaei, K., Valečková, E., Dube, F., Wall, H., Roos, S. & Ellström, P. Effects of silage inoculated with different lactobacilli on growth and survival of *Campylobacter jejuni* (manuscript submitted to *Microorganisms*)

II. Valečková, E., Ivarsson, E., Ellström, P., Wang, H., Mogodiniyai Kasmaei, K., & Wall, H. (2020). Silage and haylage as forage in slow and fast-growing broilers – effects on performance in *Campylobacter jejuni* infected birds. *British Poultry Science* 61 (4), 433-441.

III. Valečková, E., Sun, L., Wang, H., Mogodiniyai Kasmaei, K., Dube, F., Ellström, P., Ivarsson, E. & Wall, H. (2023). Intestinal colonization with *Campylobacter jejuni* affects broiler gut microbiota composition but is not inhibited by daily intake of *Lactiplantibacillus plantarum*. *Frontiers in Microbiology* 14.

IV. Eliasson, E., Sun, L., Ellström, P. & Ivarsson, E. Development of a caecal microbiota and its role in resistance to *Campylobacter jejuni* infection in broilers fed brown algal extract-supplemented diets (manuscript).

Abbreviations

AE	Algal extract
ANCOM	Analysis of composition of microbiomes
ANOVA	Analysis of variance
ASV	Amplicon sequence variant
BW	Body weight
CFU	Colony-forming units
CKMS	Crimped kernel maize silage
DM	Dry matter
dpi	Days post-infection
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
FCR	Feed conversion ratio
FI	Feed intake
GIT	Gastrointestinal tract
LAB	Lactic acid bacteria
LP256	*Lactiplantibacillus plantarum* 256
mCCDA	Modified charcoal-cefoperazone-deoxycholate agar
MIC	Minimum inhibitory concentration
PCoA	Principal coordinate analysis

PERMANOVA	Permutational multivariate analysis of variance
ppm	Parts per million
qPCR	Quantitative polymerase chain reaction
RA	Relative abundance
R-308	Ross 308
RR	Rowan Ranger
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acids

1. Introduction

Poultry farming is a significant sector within the global agricultural industry and involves rearing and management of domesticated birds for various purposes. The species reared are predominantly chickens, ducks, turkeys and geese, raised for eggs and meat. The main sectors within the industry are egg production from laying hens and meat production from selectively bred broilers (Mottet & Tempio, 2017). The broiler industry has achieved continuous growth and development in recent decades, which can be attributed to advances in genetics, nutrition, biosecurity measures and management practices focusing on growth rate optimisation, feed conversion efficiency and meat yield. In combination, these factors have led to a considerable reduction in the time required for conventional broilers to reach marketable weight, thereby enhancing overall food production efficiency to meet the high demand for poultry meat worldwide (Castro *et al.*, 2023). However, this intensive form of production has also sparked a debate about the need to set a balance between production efficiency and consideration of risks to animal welfare (Panel *et al.*, 2023). Therefore, some consumers look for broiler meat produced by different farming methods, such as free-range and organic systems (Escobedo del Bosque *et al.*, 2021). However, access by broilers to outdoor areas in such systems involves increased contact with potential pathogen reservoirs, such as wild birds, rodents and flies, posing a risk of zoonotic disease transmission (Rosenquist *et al.*, 2013).

2. Background

2.1 Broiler meat production

To meet escalating global demand for affordable protein sources, poultry meat production underwent a 10-fold increase between 1960 and 2020 (Ritchie *et al.*, 2017). Notably, this type of meat is accepted by different religions and fits with a shift in consumer preference from red to white meat among the continually expanding global population. In 2022, broiler meat production worldwide reached 102 million metric tons and represented nearly 40% of total global meat production (Statista, 2023). The USA is currently the largest chicken meat producer, with 17% of global production, followed by China, Brazil and the European Union (EU). In terms of international trading, Brazil takes the lead in global chicken meat exports (FAO, 2023).

Within the EU, Poland is the leading producer of chicken meat, contributing approximately 20% of the total output (FAOSTAT, 2023). Notably, Ukraine is currently significantly increasing its export of chicken meat to European countries, due to European free-trade measures designed to support Ukraine after the Russian invasion (AVEC, 2023).

Sweden produces approximately 110 million boilers per year (Jordbruksverket, 2023). Conventional broilers are raised on 120 farms, with an average batch size of approximately 85,000 birds. Broiler producers on these farms rear around seven batches per year (Swedish Poultry Meat Association, 2022). Broilers are processed at seven slaughter plants, where the dominant abattoir Kronfågel handles on average 55 million broilers per year (Jordbruksaktuellt, 2017).

However, broiler as a popular meat option is also contributing to the spread of common human foodborne pathogens (Broom & Kogut, 2018), such as *Campylobacter* and *Salmonella*. Therefore, investigating methods to enhance the resilience of broilers to pathogens and zoonotic bacteria arising within production systems is of interest to the scientific community. The modern conventional chicken meat industry involves hatcheries that supply day-old chicks to farms, where broilers are raised under strictly maintained conditions. At approximately 35 days of age, broilers are transported to a slaughterhouse and a sanitisation process is implemented on-farm to ensure the hygiene and biosecurity of the houses after each production cycle. Regardless of this streamlined controlled system and despite long-standing research efforts, broilers are still the main source of human *Campylobacter* infections in many countries.

2.2 Campylobacter

Campylobacter spp. are gram-negative bacteria, typically appearing as curved or comma-shaped rods exhibiting high motility through either unipolar or bipolar flagella (Garrity *et al.*, 2005). The genus *Campylobacter* currently comprises 32 species and nine subspecies, but only some of these are of significance to human health (Costa & Iraola, 2019). The two species most frequently associated with human disease are *Campylobacter jejuni* (88% of cases) and *Campylobacter coli* (10% of cases) (EFSA, 2020). *Campylobacter jejuni* is a thermophilic species requiring a microaerobic environment and incubation temperatures of 37-42 °C. In broilers, *Campylobacter* inhabits the mucus layer above the epithelial cells predominantly in the caeca and small intestine, but it may also be detected in other areas of the gut (Beery *et al.*, 1988).

2.2.1 Broiler gut colonisation

Wild birds have been identified as an important natural host for *C. jejuni* and a possible source of transmission to broiler production (Waldenström *et al.*, 2002). Rodents and insects serve as vectors (Mourkas *et al.*, 2022), disseminating this pathogen through faecal contamination into the surrounding environment, surface water and feed on farms (Hald *et al.*, 2004). Return of flies, beetles and mice to broiler houses after the facility has been cleaned and disinfected may contribute to the recurrence of

Campylobacter in subsequent broiler batches (Nichols, 2005; Hald *et al.*, 2004; Berndtson *et al.*, 1996). Thus while sanitisation can lower the levels of *C. jejuni* organisms, completely eradicating the pathogen from contaminated farms remains challenging, and subsequent outbreaks can occur on the same farm (Hansson *et al.*, 2007). Furthermore, higher prevalence of *Campylobacter* spp. in broilers is reported during warmer months, likely due to the increased ventilation leading to more insects entering broiler houses (Sandberg *et al.*, 2015).

It is well established that broilers rapidly accumulate high numbers of *C. jejuni* in the caecal contents (load around 1×10^9 colony-forming units (CFU) per g) within three days after infection (Shanker *et al.*, 1990). Since broilers are coprophagic, faecal shedding is an important mechanism for within-flock transmission of infection. In conventional intensive systems with a rather short production cycle, it is generally accepted that once broilers are infected, the infection persists until the time of slaughter (Connerton *et al.*, 2018).

2.2.2 Campylobacteriosis

While *C. jejuni* is generally acknowledged as a commensal organism in broilers and is not typically associated with clinical symptoms (Hermans *et al.*, 2012), it is known to cause a disease called campylobacteriosis in humans. This acute diarrhoeal disease is the most commonly reported zoonosis within the EU (EFSA and ECDC, 2023). The European Food Safety Authority (EFSA) has categorised *Campylobacter* as a high-priority hazard in poultry, given that 50-80% of human infections in the EU are linked to consumption of contaminated poultry products. *Campylobacter* infections often arise from mishandling of raw contaminated poultry products or consumption of undercooked poultry meat.

Campylobacteriosis in humans can result from a low infection dose, and besides diarrhoea, symptoms such as fever, headache, vomiting and abdominal pain may occur (Teunis *et al*., 2018). Onset of symptoms typically occurs two to five days after exposure, with most infected individuals recovering within a week. However, according to the European Centre for Disease Prevention and Control (EFSA and ECDC, 2023), children and individuals with a compromised immune system may experience abrupt infection, potentially developing into post-infectious complications such as gastrointestinal and joint disorders or immune-mediated neurological conditions, such as Guillain-Barré syndrome.

2.2.3 Control strategies

Developing strategies to reduce *Campylobacter* spp. in the gut of broiler chickens at farm level is crucial for minimising the risk of carcass contamination during slaughter, thereby eliminating a potential transmission route for this pathogen to humans (Hermans *et al.*, 2012). The EFSA is responsible for monitoring *Campylobacter* in the EU and the European Economic Area countries, but actual responsibility for conducting *Campylobacter* surveillance rests with individual member countries. In accordance with process hygiene criteria established by EU regulations in 2018 (EU Commission, 2005), many European countries routinely test neck skin samples for *Campylobacter* (Table 1). Additionally, northern European countries have implemented their own national *Campylobacter* surveillance plans. For instance, Sweden, Norway and Finland test caeca at slaughterhouses, while in Denmark surveillance involves testing cloaca and leg skin samples at slaughterhouses, along with meat samples at retail level (Olsen *et al.*, 2024). Prevalence of *Campylobacter* in Swedish commercial broiler production (and in several other Nordic countries) is markedly lower than in many other European countries (Table 2). In addition, there has been a considerable decrease in *Campylobacter*-positive flocks in Sweden over the past few years. Since Sweden has a zero-tolerance policy for *Salmonella* in chicken meat and other food sources, the strategies implemented to prevent *Salmonella* are believed to have contributed to the low prevalence of *Campylobacter*-positive flocks, as biosecurity is of high importance for both.

The latest report from EFSA estimates that a 3-$\log_{10}$ reduction in broiler caecal concentrations of *C. jejuni* can reduce the relative EU-wide risk of human campylobacteriosis attributable to broiler meat by 58%. According to that report, the most promising control options identified in models incorporating scientific data are as follows: vaccination, use of feed and water supplements, discontinuation of bird thinning (partial depopulation), use of a limited panel of well-trained staff, implementation of hygiene anterooms, and allocation of designated tools for each broiler house (EFSA, 2020). It is worth mentioning that development and testing of effective vaccines are still ongoing (Pumtang-On *et al.*, 2021), but vaccination alone is unlikely to solve the problem unless there is a high level of biosecurity and management in place.

Table 1. Percentage of neck skin samples from slaughterhouses exceeding the high-level *Campylobacter* contamination threshold (>1000 CFU/g). Data retrieved from EFSA and ECDC, 2023.

	2020		2021	
Country	No. of samples	Positive (%)	No. of samples	Positive (%)
Denmark	985	7.0	1,150	7.5
Estonia	260	1.9	260	0.0
Germany	5,556	7.5	6,604	7.7
Finland	595	0.2	585	0.2
France	15,481	28.5	16,357	26.8
Iceland	693	0.0	773	0.5
Italy	6,591	10.7	5,591	8.3
Norway	525	0.0	1,620	0.1
Poland	-	-	1,365	8.0
Portugal	3,601	12.6	3,528	14.8
Serbia	125	38.4	-	-
Sweden	907	0.8	1,046	1.4

Table 2. Prevalence of *Campylobacter* in broiler flocks across Nordic countries with a national surveillance program. Adapted from Olsen *et al.*, 2024.

		Proportion positive (%*)				
Country	Sample type	2017	2018	2019	2020	2021
Denmark	Cloacal swab	16.6	24.6	22.7	20.2	19.5
Finland	Cecal sample	1.5	2.9	2.5	4.4	7.2
Iceland	Faecal swab	3.1	2.2	1.9	0.6	1.0
Norway	Cecal sample	6.9	6.3	5.1	6.1	5.8
Sweden	Cecal sample	10.7	8.7	4.6	4.6	5.3

*proportion positive is determined by the country-specific threshold of CFU/g in the tested sample

2.3 Alternative broiler production systems

Achieving low flock prevalence of *C. jejuni* is more complicated in free-range and organic broiler production systems. In contrast to conventionally reared broilers, free-range and organic broilers are provided with access to outdoor areas, increasing the likelihood of contact with *Campylobacter*

carriers such as vermin and insects. Furthermore, organic broilers have a longer lifespan, meaning a prolonged exposure period for potential encounters with *C. jejuni*. In addition, stricter legal regulations apply to organic production systems, limiting the use of additives, treatments and disinfectants (Lassen *et al.*, 2022).

Organic animal farming aims to promote the health and well-being of animals, giving attention to their behavioural needs (Vaarst & Alrøe, 2012). Current EU regulations on organic broiler production mandate *e.g*. outdoor access for the birds and lower stocking densities (Commission Regulation (EC) 889/2008), in order to provide them with opportunities to engage in natural behaviours such as foraging and dust bathing. Broilers are required to have daily access to forage during periods without outdoor access (Commission Regulation (EC) 889/2008). Since suitable hybrids must be selected for this system, fast-growing broilers have been replaced by slower-growing hybrids. Rowan Ranger and Hubbard, both slower-growing hybrids, entered the commercial market in Sweden in 2014 and 2016, respectively. This led to an increase in the number of organic broiler farms between 2015 and 2017 (Göransson *et al.*, 2021). Despite this increase, in 2022 only 1% of total Swedish broiler production (equivalent to 145,300 birds) was organic production (Swedish Board of Agriculture, 2022).

2.4 Diet supplementation and alternative diets

Several feed supplements have been suggested to enhance the overall robustness of birds and influence the gut microbiota of broilers, thereby reducing the prevalence of *C. jejuni* in the gastrointestinal tract (GIT) of broilers.

2.4.1 Probiotics

Probiotics, classified as live microorganisms, may be incorporated into the diet of animals as feed supplements and provide beneficial properties to the host, primarily through their action in the GIT (Abd El-Hack *et al.*, 2020). In various studies, probiotics have been demonstrated to have a positive effect on broiler production performance. In a study conducted by Karimi Torshizi *et al.* (2010), a multi-strain probiotic supplement was administered in water to Ross 308 broilers. The results showed increased body weight (BW), higher feed intake (FI) during the starter period and lower feed conversion ratio

(FCR) in groups receiving the probiotic compared with the control group. The results also demonstrated that the method of probiotic administration significantly influenced the efficacy, with provision through drinking water being identified as highly effective (Karimi Torshizi *et al*., 2010).

Single-strain probiotic species, including species of *Bifidobacterium*, *Bacillus*, *Enterococcus*, *Lactobacillus* and *Streptococcus*, have been shown to have positive effects not only on broiler performance, but also in modulation of the gut microbiome and inhibition of pathogens (Krysiak *et al.*, 2021; Prabhurajeshwar & Chandrakanth, 2019; Neal-McKinney *et al.*, 2012). In the context of lactic acid bacteria (LAB), the following mechanisms have been identified:

(i) Niche occupation and shifts in the gut microbiota composition leading to competitive exclusion (Stecher *et al.*, 2010), where the probiotic microbes may serve as a barrier, restricting the occurrence of pathogenic microbes. Restriction may occur through physical exclusion, where pathogens are prevented from binding to specific sites in the gut. Alternatively, it can occur indirectly through the microbiota occupying essential resources or niches (Nurmi & Rantala, 1973). While the precise mechanisms of competitive exclusion have not been fully elucidated, it remains the most effective method identified so far for preventing *Salmonella* colonisation in live birds (Oakley *et al.*, 2014).

(ii) Production of organic acids, since *Campylobacter* exhibits sensitivity to environmental stress when outside its host and susceptibility to disinfectants, oxygen exposure, osmotic stress and acidity (Park, 2002; Trachoo & Frank, 2002; Doyle & Roman, 1982; Blaser *et al*., 1980). However, despite its fragility during *in vitro* settings, *Campylobacter* appears to be adapted to surviving the highly acidic conditions of the stomach during its passage to the lower intestinal tract (Axelsson-Olsson *et al.*, 2010).

(iii) Production of antimicrobial metabolites and bacteriocins (Messaoudi *et al.*, 2012), where certain strains of LAB have the ability to naturally synthesise antimicrobial peptides or proteins that exhibit activity against both Gram-positive and Gram-negative bacteria. The inhibitory effect of these compounds on relevant pathogenic bacteria is well-documented in *in vitro* studies (Newstead *et al.*, 2020; Gabrielsen *et al.*, 2014; Perez *et al.*, 2014; Cui *et al.*, 2012).

Specific *Lactobacillus* species, commonly present in poultry gut microbiota, have been shown to mitigate *Campylobacter* colonisation through the mechanisms outlined above. For example, a bacteriocin (OR-7), derived from a *Lactobacillus salivarius* strain, exerts a considerable inhibitory effect on *C. jejuni* colonisation within the broiler intestinal tract when integrated into the feed (Stern *et al.*, 2006). In a study by Saint-Cyr *et al.* (2017) where Ross broilers were administered an oral gavage of *Lactobacillus salivarius* SMXD51 (10^7 CFU) 24 hours after hatch, the treated group showed a substantial reduction in *C. jejuni* loads in the gut at 14 days of age (0.82 log reduction) and 35 days of age (2.81 log reduction) compared with control birds. The primary inhibitory mechanism was attributed to the bacteriocin (SMXD51) in that study (Saint-Cyr *et al.*, 2017). A study by Šimunović *et al.* (2022) found that adding *Bacillus subtilis* PS-216 spores to drinking water resulted in a reduction in *C. jejuni* colonisation in broilers, and a simultaneous improvement in weight gain.

Despite the promising reports of probiotics in reducing *Campylobacter* colonisation in broilers, numerous studies have reported disparate outcomes (Ty *et al.*, 2022; Mortada *et al.*, 2020; Robyn *et al.*, 2013; Robyn *et al.*, 2012), with a notable proportion of studies demonstrating limited replicability.

2.4.2 Forage

As mentioned, organically reared birds in the EU must be given the opportunity to range outdoors and daily access to forage, which can be defined as feedstuffs rich in insoluble fibre (Choct, 2015). However, despite this requirement for forage access, recommendations on quantity, quality and predicted intake are lacking for both organic and conventional broilers. Insoluble fibre, a compound resistant to digestive enzymes, was formerly seen as a diluent in poultry diets, potentially lowering feed intake and nutrient digestibility (Rougière & Carré, 2010; Sklan *et al.*, 2003). However, several publications suggest that a moderate amount of insoluble fibre can have a positive effect on nutrient availability (Svihus, 2011), volume of gizzard contents (Hetland *et al.*, 2005) and digestive tract development (González-Alvarado *et al.*, 2008).

In temperate regions, forage is often stored in the form of silage. This fermented product resulting from anaerobic storage has a high moisture content (50-70%) and low pH (around 4), and is rich in lactic acid bacteria (LAB), which comprise approximately 10^7 CFU/g of fresh matter. Silage is

generally viewed as a key feedstuff for ruminants, but there is growing interest in incorporating silage and other forage into the diet of monogastric animal diets, as observed on some organic broiler farms (Crawley, 2015). Fermented feeds with low pH and high LAB counts have been shown to have some potential to reduce susceptibility to *C. jejuni* colonisation in Ross broilers by enhancing upper intestinal barrier function (Heres *et al.*, 2004). Hence, it can be speculated that silage, with its low pH, diverse LAB species and elevated fibre content, has the potential to reduce the abundance of *C. jejuni* in the gut of broiler chickens.

In the Nordic countries, forages are also commonly stored anaerobically as a fermented product known as haylage. This preservation method involves wrapping wilted forage with 30-50% water content in plastic foil. Unlike silage, haylage typically has lower LAB content and higher pH due to a restricted fermentation (Müller, 2005).

2.4.3 Prebiotics

Unlike probiotics, prebiotics are not microorganisms. Instead, they are nutritional compounds that are not digested by the host, but serve as a source of nutrients for the gut microorganisms (*e.g. Bifidobacteria* and *Lactobacillus*), promoting their proliferation in the GIT (Gibson & Roberfroid, 1995). This modification in microbiota composition, along with modulation of fermentation, has been shown to enhance broiler performance (Froebel *et al.*, 2019) and is suggested to hinder establishment of foodborne pathogens such as *Campylobacter* and *Salmonella* in the gut (Ricke, 2021).

Brown macroalgae, also known as seaweed, are an attractive source of biomass for novel feed components (Øverland *et al.*, 2019). *Saccharina latissima*, a member of the *Laminariaceae* family, is a brown macroalgal species rich in complex polysaccharides, such as alginate and laminarin (Michell *et al.*, 1996). These polysaccharides have been proposed as novel sources of bioactive compounds (Sweeney & O'Doherty, 2016), with laminarin identified for its potential prebiotic properties (Cherry *et al.*, 2019).

Research on laminarin-rich extracts from *Saccharina* spp. as a feed additive has been conducted in livestock, particularly in pigs (O'Shea *et al.*, 2014; Leonard *et al.*, 2011) and poultry (Maiorano *et al.*, 2017; Sun *et al.*, 2016). In one study, inclusion of 300 ppm of laminarin in a broiler diet was found to promote a beneficial profile of the gastrointestinal microbiota, with

an increase in absolute and relative abundance of *Bifidobacterium* spp. (Venardou *et al.*, 2021).

2.5 Broiler gut microbiota

2.5.1 Broiler digestive tract

The digestive tract of broilers consists of the crop, proventriculus, gizzard, small intestine (including the duodenum, jejunum and ileum) and large intestine (paired caeca and a short colon), which connects to the cloaca (Figure 1). The crop, an enlarged portion of the alimentary tract, is involved in storage and moistening of feed (Rodrigues & Choct, 2018). The feed is then mixed with pepsin, hydrochloric acid and mucus in the proventriculus, crushed in the gizzard, and finally moved to the small intestine. Within the small intestine, the duodenum, jejunum and ileum are crucial for nutrient absorption. The caeca have metabolism-related functions, acting as a key region for bacterial fermentation of nondigestible carbohydrates (Svihus *et al.*, 2013). They are also believed to play an important role in broiler gut health, but understanding of their role in maintenance of gut health and modulation of the gut microbiota remains incomplete.

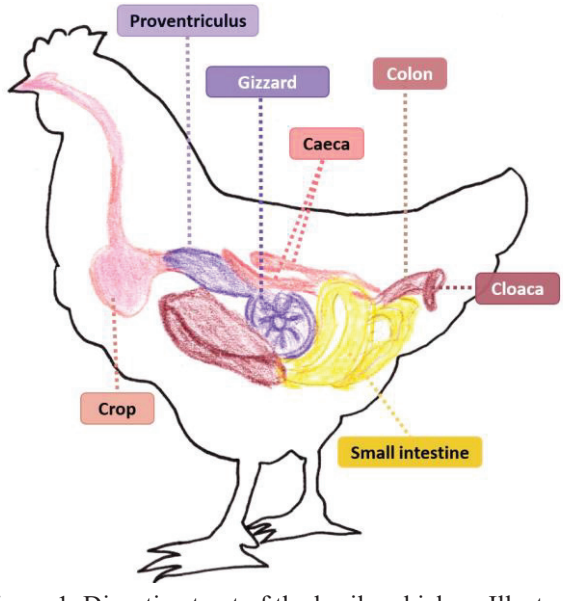

Figure 1. Digestive tract of the broiler chicken. Illustration by Alena Valečková.

2.5.2 Gut microbiota development

The GIT microbiota in broiler chickens is a complex microbial community that plays an important role in nutrition, defence against pathogens and immune system development (Shang *et al.*, 2018; Stecher & Hardt, 2008). In modern commercial birds, the microbiota is considerably different from that of their wild ancestors, native jungle fowl, where chicks are in contact with hens and the nest environment and obtain their microbiota from these. Establishment of the gut microbiota in commercial broilers is profoundly impacted by the surrounding environment (Zhou *et al.*, 2021; Stanley *et al.*, 2013). It is generally considered to be fully established within the initial weeks of life, achieving high microbial density that persists during the life of the broiler, although adapting continuously to environmental changes and the physiological state of the host (Haberecht *et al.*, 2020). Importantly, understanding the dynamics of the intestinal microbial community is essential for developing feed supplements and implementing dietary changes that can improve broiler health and performance (Sugiharto, 2016).

2.5.3 Gut microbiota composition

The microbial composition within the GIT of broilers is influenced by the specific metabolic functions performed in each section (Zhou *et al.*, 2021; Shang *et al.*, 2018). The crop harbours 10^8 to 10^9 CFU/g bacteria, usually dominated by lactobacilli (Abbas Hilmi *et al.*, 2007). In the gizzard, the concentration of bacteria (mainly lactobacilli, enterococci, enterobacteria and coliform bacteria) is around 10^7 to 10^8 CFU/g, but bacterial fermentation activity is lower due to the low pH. The small intestine is mainly colonised (10^8 to 10^9 CFU/g) by acid-tolerant, facultative anaerobes such as clostridia, streptococci, enterobacteria and lactobacilli, which can withstand the enzymes, high oxygen pressure and bile salts present in the duodenum (Soumeh *et al.*, 2021). Microbiota composition and complexity considerably increase in distal parts of the intestinal tract (caecum and colon). However, due to the physiology of the chicken intestinal tract, the colonic microbiota is variable and may resemble either the ileal or caecal microbiota (Rychlík, 2020). The most densely colonised microbial habitats in broilers are the paired caeca, with microbial density of around 10^{11} to 10^{12} bacterial cells per gram (Rinttilä & Apajalahti, 2013). They are primarily colonised by obligate anaerobes, including *Clostridium*, *Bacteroides* and *Ruminococcus*, or facultative anaerobes such as *E. coli*, *Streptococcus* and *Enterococcus*.

In free-range and organic production settings, the high biosecurity measures employed in conventional production are not possible as the broilers are exposed to the outdoor environment, enabling encounters with soil, grass and diverse plant microbiota, together with potential exposure to the microbiota of rodents, wild birds and other animals (Ocejo *et al.*, 2019). Thus, Varriale *et al.* (2022) observed significant variations in the composition of caecal microbiota in Hubbard broilers before outdoor access (28 days of age) and after access (56 days of age), where the group with outdoor access exhibited a more diverse microbial community, indicating the impact of environmental factors on the microbiota. However, it should be noted that those authors did not address the impact of age on caecal microbiota complexity. In a study by Ocejo *et al.* (2019), age was identified the strongest factor influencing the composition of caecal microbiota, surpassing the impact of diverse breeds or management systems.

There are currently only a few studies in the published literature describing differences in the caecal microbiota of broilers between *Campylobacter*-positive and *Campylobacter*-free flocks, especially within commercial settings. In one such study, by Pang *et al.* (2023), comparative analysis of the microbiota revealed an increase in species richness and phylogenetic diversity in *Campylobacter*-positive flocks, along with a higher abundance of several bacteria in *Campylobacter*-free flocks. Those authors suggest that identification of specific bacteria exhibiting higher abundance in *Campylobacter*-free broilers may be useful in the development of tailored probiotic formulations.

3. Aims and Objectives

Conventional broiler production in Sweden has a low prevalence of *Campylobacter*, but higher incidences are still common in many EU countries, particularly in organic production. Given that *C. jejuni* poses a risk to public health, identifying ways to reduce its levels in the broiler gut is of high importance. Various alternative diets and dietary supplements, such as lactic acid bacteria or algal saccharides with bioactive properties, could be promising components of a comprehensive solution to this challenge. The overall aim of this thesis was to assess the effects of such components on *C. jejuni* colonisation in broilers, on caecal microbiota and on production performance. Specific aims of the work described in Papers I-IV were to:

- Determine whether specific lactobacilli strains in silage extracts can inhibit *C. jejuni* growth *in vitro* and identify the main inhibition mechanism (Paper I)

- Evaluate the effects of daily consumption of silage, haylage and *Lactiplantibacillus plantarum* 256 (*L. plantarum* 256) on the production performance and organ development of broilers (Paper II)

- Examine the impact of daily intake of *L. plantarum* 256 on caecal microbiota composition and *C. jejuni* loads in the faeces and caeca of experimentally colonised broilers (Paper III)

- Assess the impact of adding laminarin-rich algal extract to broiler diets on development of the caecal microbiota and *C. jejuni* loads in faeces of experimentally colonised broilers (Paper IV).

4. Methodology

To obtain guidance for subsequent *in vivo* feeding trials, an *in vitro* study was conducted to evaluate the most promising lactobacilli strain for silage preparation with the potential to inhibit *C. jejuni* growth (Paper I). The most effective strain identified, *L. plantarum* 256, was then used in animal experiments (*L. plantarum* 256 study; Papers II and III). The impact of algal extract (AE) on caecal microbiota development and *C. jejuni* levels in broilers was examined in a separate study (AE study; Paper IV).

All animal experiments described in this thesis (Papers II-IV) were performed at the Swedish Livestock Research Centre, Swedish University of Agricultural Sciences, Uppsala, and were approved by the committee for animal ethics of the Uppsala region (approval number 5.8.18-16271/2017 for *L. plantarum* 256 study and no. 5.8.18-10572/2019 for the AE study). The birds used in the studies were Aviagen's Ross-308 hybrids (Papers II-IV), the most widely used fast-growing hybrid for chicken meat production worldwide, and Rowan Ranger hybrids (Papers II and III), the slower-growing hybrid used in organic production in Sweden.

In all *in vivo* experiments, the broilers were housed in raised pens (1.5 m x 0.75 m) with fresh wood shavings as litter. The experimental diets were based on organic (Papers II and III) or conventional pellets (Paper IV), which were manually distributed. Water was provided in bell drinkers (Papers II and III) or nipple drinkers (Paper IV). For the experimental *C. jejuni* challenge, colonisation was performed via water in bell drinkers (Papers II and III) or intra-oesophageal inoculation (Paper IV). A detailed description of the materials and methods used in the experiments is provided in Papers I-IV. A brief overview is given below, along with remarks on the methods.

4.1 Paper I

To assess the inhibitory potential of grass and maize silage extracts on *in vitro* growth of *C. jejuni*, grass-clover and whole-crop maize samples with 40% dry matter were inoculated with 5 $\log_{10}$ CFU/g forage of one of the following strains: *Lactiplantibacillus plantarum* 256, *Lactiplantibacillus plantarum* E-78076 and *Limosilactobacillus reuteri* DSM 20016. In addition, *Limosilactobacillus reuteri* supplemented with 5% glycerol was tested. The inoculated samples (70 g) were ensiled in glass tubes for 45 days, followed by extraction of silage juice. The inhibitory effect of the silage extracts on growth of *C. jejuni* was evaluated in a minimum inhibitory concentration (MIC) assay. Untreated, heat-treated and neutralised preparations of the silage extracts were also compared in spot diffusion growth inhibition tests on soft agar, to identify the primary inhibition mechanism.

4.2 Paper II and Paper III

To evaluate treatment effects on broiler performance (Paper II) and on gut microbiota composition and *C. jejuni* colonisation (Paper III), two experiments were carried out in parallel, each involving 160 one-day-old broilers. One experiment, with Ross 308 (R-308) broilers, lasted 42 days, while the other experiment, with Rowan Ranger (RR) broilers, lasted 63 days. Broilers of each hybrid were divided into four treatment groups: inoculated silage, haylage, *L. plantarum* 256 provided via water (LPW) and an untreated control. Grass for the silage was inoculated with *L. plantarum* 256 (10^8 CFU/g fresh matter) during baling, while haylage was used in the study as forage similar to silage, but without any inoculum. Silage and haylage were provided as total mixed rations in mixtures of 85% pellets and 15% of the respective forage (on dry matter (DM) basis). The LPW and control groups received feed without forage inclusion. For the LPW group, *L. plantarum* 256 (10^7 CFU/mL) was administered by direct supplementation in the drinking water, while the other groups received unsupplemented water. The birds were challenged with *C. jejuni* #65 via water (10^6 CFU/mL) at three weeks of age in R-308 and at four weeks of age in RR. Enumeration of LAB in silage and haylage material was performed prior to the experiments and once per month thereafter.

For assessment of production performance (Paper II), feed intake (FI), forage and pellet intake and body weight (BW) were recorded weekly and used to calculate feed conversion ratio (FCR). Collection of caecal samples were performed at -1, 3 and 20 days post-infection (dpi) for R-308 and -1, 3, 13 and 34 dpi for RR (Figure 2) and grading of gizzard surfaces were performed at -1 and 20 dpi for R-308 and -1 and 13 dpi for RR. The caecal samples were then subjected to analysis of microbiota composition using 16S rRNA sequencing and to determination of *C. jejuni* caecal loads via quantitative polymerase chain reaction (qPCR) (Paper III). Samples of faecal matter were collected on multiple occasions (Figure 2) and the loads of *C. jejuni* in faeces were determined by plate counts. Note that the *C. jejuni* loads in faecal matter reported in Papers II and III were determined on samples collected from the same experiment. In Paper II, *C. jejuni* levels at the end of trial were reported to justify the incorporation of *C. jejuni* challenge into the experimental design. Comprehensive results from faecal plating throughout the entire trial, along with caecal qPCR analysis, are detailed in Paper III.

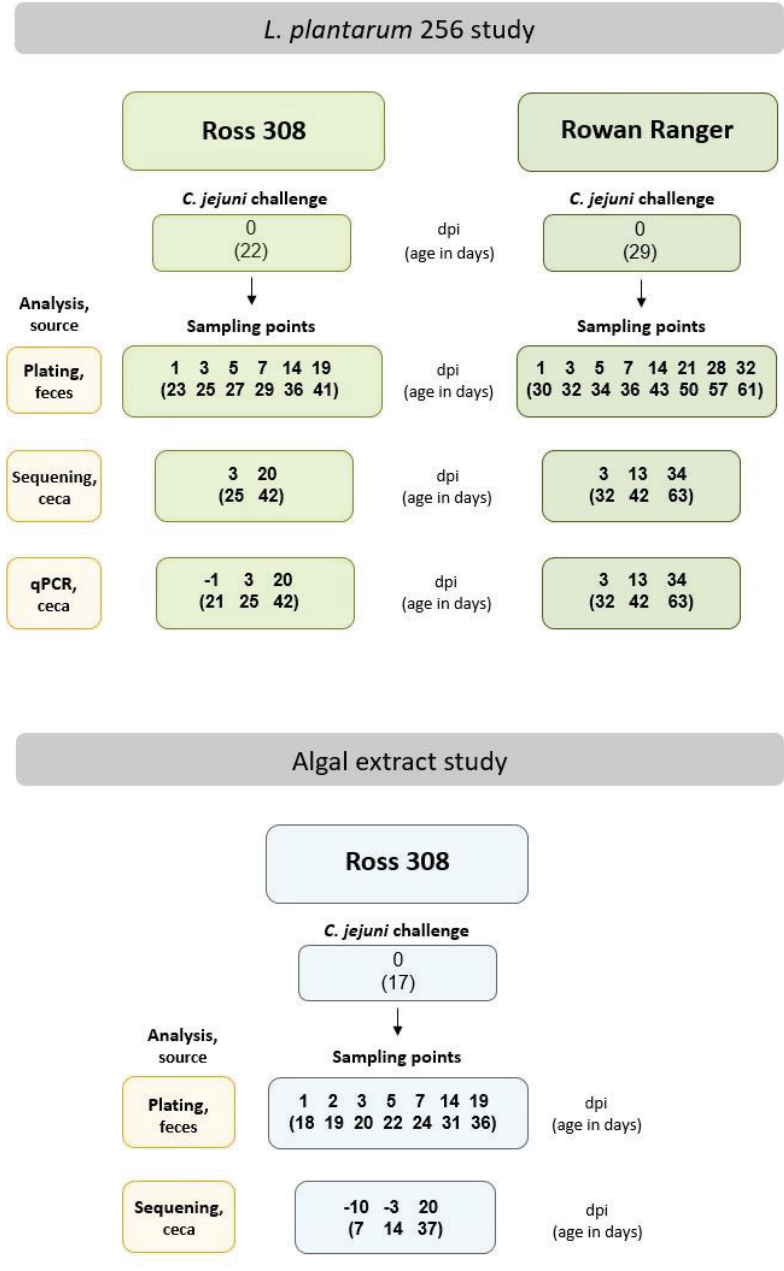

Figure 2. Flowchart showing *Campylobacter jejuni* challenge occasions and subsequent sampling points in broiler experiments involving inclusion of *Lactiplantibacillus plantarum* 256 and addition of algal extract (AE).

4.3 Paper IV

To investigate the impact of supplementing broiler feed with a laminarin-rich extract derived from the brown algae *Saccharina latissima* on the development of caecal microbiota in broilers and on broiler resistance to intestinal colonisation with *C. jejuni*, a total of 255 R-308 chicks were assigned to 24 experimental pens in groups of ~10 broilers. In a 37-day-long experiment, the broiler facility was divided into two sections, each comprising 12 pens. In each section, two chick treatment groups were established: one received a basal diet formulated according to R-308 nutrient requirements, while the other received a basal diet supplemented with 725 ppm AE (Figure 3). The diet was optimised to contain 290 ppm of laminarin. At 17 days of age, group size was reduced to four broilers per pen and chicks were subjected to intra-oesophageal inoculation with two *C. jejuni* strains (of broiler and song thrush origin). Faeces sampling was performed on multiple occasions for *C. jejuni* enumeration by plate counts (Figure 2). Caecum sampling on two birds per pen was performed at 7, 14 and 37 days of age, followed by 16S rRNA amplicon sequencing.

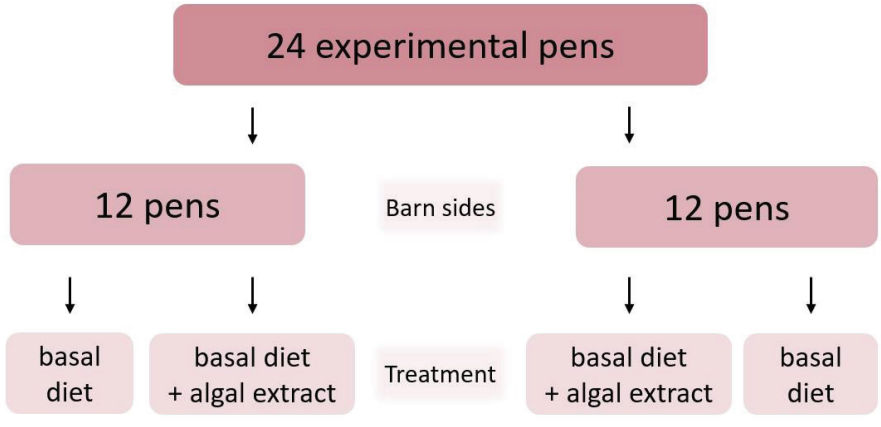

Figure 3. Flowchart summarising steps in an experiment involving addition of algal extract (AE) to the diet of broiler chickens.

4.4 Remarks on methodology

4.4.1 Pathways of *Campylobacter* colonisation

Within the research community, various methods are employed for experimental *C. jejuni* colonisation, such as oral gavage, oral inoculation via feed and water, cloacal swab inoculation or litter contamination, based on specific research aims. Direct inoculation through oral gavage (10^2 CFU/mL) was applied in the AE study in Paper IV, as it is currently the main approach used for experimental *C. jejuni* challenge due to its precise dosing capabilities (Shanker *et al.*, 1988). In the *L. plantarum* 256 study in Papers II and III, on the other hand, the broilers were orally challenged by inoculation of a fixed dose (10^7 CFU/mL) into the drinking water. The intention with latter approach was to replicate a more 'natural' mode of colonisation, aiming to simulate conditions in organic production settings.

4.4.2 Time of *Campylobacter* colonisation

Maternal antibodies received from the hen are believed to play a protective role against *Campylobacter* in young broiler chickens, which usually test negative for *Campylobacter* during the initial 14-21 days of rearing in conventional housing systems (Sahin *et al.*, 2003). Therefore, in the *L. plantarum* 256 study in Papers II and III, the challenge to R-308 chickens, selected as the typical broiler type in conventional production settings, was initiated at three weeks (22 days) of age. This time was chosen to mitigate the risk of unsuccessful early colonisation owing to residual maternal immunity. The RR birds in Papers II and III were colonised at four weeks (29 days) of age, corresponding to the time when birds in organic production are given access to outdoor areas and encounter *C. jejuni* reservoirs. In the AE study in Paper IV, the R-308 birds were inoculated at 17 days of age, following the experimental design.

4.4.3 Analysis of faeces samples

The *C. jejuni* loads in faecal matter (Papers III and IV) were assessed through faecal culture and colony counts on modified charcoal cefoperazone deoxycholate (mCCDA) agar plates. This agar is designed for isolating, detecting and enumerating *Campylobacter spp.* from diverse sources (food, animal feed, environmental samples) and contains the agents deoxycholate

and cefoperazone to selectively inhibit accompanying bacteria, yeasts and moulds. Faeces samples were collected from random birds one day before the *C. jejuni* challenge, to ensure that the birds were initially negative for *Campylobacter*. Faeces samples were collected regularly throughout the experiment (Figure 2), and were re-suspended in Luria-Bertani medium with glycerol, followed by vortexing and centrifugation to pellet crude faecal matter. A 100 µL aliquot of the supernatant was withdrawn, serially diluted in a 10-fold series and plated on mCCDA. After plating, incubation was performed at 42 °C (~30 h) under microaerobic conditions, followed by colony counting on the plate corresponding to a dilution yielding approximately 100 CFU per plate.

4.4.4 Analysis of caecal samples

Quantification of *C. jejuni* in caecal samples (Paper III) was performed using a qPCR method with the capability to detect both viable and non-viable cells, thereby offering a more comprehensive assessment of caecal microbial load. A primer pair adopted from Atterby *et al*. (2018) targeting the d65_1178 gene, specific to *C. jejuni* strain #65 and its sequence type ST-104 (ST-21 CC), was used. Reactions were run in triplicate, where the reaction mixture comprised SsoAdvanced Universal SYBR Green Supermix, forward and reverse primer, and the template.

Extraction of DNA from caecal digesta samples was performed in the same way in the *L. plantarum* 256 and AE studies (Papers III and IV). In brief, 400 µL of ASL lysis buffer (Qiagen, Germany) were added to the thawed sample and homogenised. Then 120 µL suspension were subjected to bead beating in a Precellys evolution homogeniser (Bertin Technologies SAS, France) at 8000 rpm for 2 × 60 s, with 30 s pause. After centrifugation, the supernatant was used for DNA extraction using the EZ1 Advanced XL instrument (Qiagen, Germany) according to the manufacturer's instructions.

4.4.5 Gut microbiota analysis

After DNA extraction from caecal samples (Paper III and IV), the samples were sent to Novogene for library preparation and sequencing. In brief (see Papers III and IV for more details), the V3-V4 region of the 16S rRNA gene was amplified with the primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTAT CTAAT) in Paper III, while the V4 region of the 16S rRNA gene was amplified with the primers 515F (GTGCCAGC

MGCCGCGGTAA) and 806R (GGACTACHVGGTWTCTAAT) in Paper IV, using the Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Sequencing was performed on the Illumina Miseq PE 250 platform (Paper III) or Illumina NovaSeq 6000 PE250 platform (Paper IV). Bioinformatics processing was performed on the raw reads from the sequencing as described in Sun *et al.* (2022), with the following two modifications: (i) truncation length of 221 bp for both forward and reverse reads; and (ii) generalised UniFrac distance matrix (alpha = 0.5) was generated using the QIIME2 diversity plugin (Bolyen *et al*., 2019). The amplicon sequence variants (ASV) obtained were assigned taxonomy by comparison to the SILVA SSU Ref NR 99 132 database (Paper III) or the SILVA SSU Ref NR 99 138 database (Paper IV). Further analyses of the sequenced data involved determination of bacteria relative abundance at phylum and genus level (Paper III) or ASV level (Paper IV), rarefaction curves of observed ASVs and principal coordinate analysis (PCoA) of generalised UniFrac distance matrix.

The universal presence of the 16S rRNA gene in all bacteria makes 16S rRNA sequencing a valuable tool for investigating composition and diversity of microbial communities (Kamble *et al.*, 2020). The 16S rRNA gene contains both conserved and highly variable regions, where hypervariable regions contain sequence variations that are unique to specific microbial groups, allowing for differentiation at various taxonomic levels (Kamble *et al.*, 2020). Sequencing one or two hypervariable regions offers a fast and cost-effective alternative to analysis of the entire 16S gene. However, this approach is constrained by its ability to provide taxonomic classification mainly at genus level, with species-level identification occurring rarely (Gupta *et al.*, 2019). To obtain higher resolution, other sequencing methods such as PacBio or nanopore sequencing (Szoboszlay *et al.*, 2023) are more suitable, as they can detect a greater part of the microbiota community than 16S sequencing and identify less abundant taxa to a higher degree (Durazzi *et al.*, 2021). However, those methods are more costly and require more advanced bioinformatics resources. Moreover, 16S rRNA sequencing can determine relative abundance of all bacteria in a sample, and is thus a powerful method that provides reliable and rapidly obtained information (Gupta *et al.*, 2019). Thus the method was employed in the studies described in this thesis.

4.5 Statistics

To assess the effect of treatments on silage quality parameters (Paper I), the General Linear Model (Minitab, LLC, 2021) was used. The Tukey method was then applied for pairwise comparisons, to identify significant differences between treatment pairs. To compare the number of wells exhibiting complete growth inhibition of *C. jejuni* in the MIC assay, Fisher's exact test (GraphPad Prism) was chosen due to its ability to identify non-random associations between categorical variables.

Productivity measures (BW, FI, FCR) were analysed by the Proc Mixed Procedure (SAS), since this method accounted for variability between treatment groups and within pens, incorporating both fixed (treatment) and random factors (pen) in the model. To assess the treatment effects on gizzard surface condition, a scoring system ranging from 1 to 4 was used, where scores 1 to 3 indicated poor condition and 4 indicated good condition. The analysis utilised a binary logistic model (Glimmix procedure), treating surfaces as a categorical variable with outcomes: good (0) or not good (1). A similar model was applied to assess whether the treatments influenced bird mortality with binary outcomes: mortality occurred (1) or not (0). Plating results were evaluated by one-way ANOVA test (GraphPad Prism), where means of *C. jejuni* levels present in the faeces were compared among treatment groups.

To analyse and interpret the faecal plate count and caecal qPCR data (Paper III), a mixed-effect linear model (Proc Mixed procedure in SAS) with appropriate adjustments for repeated measures and random effects was used. Caecal microbiota diversity was analysed by the q2-diversity plugin. To compare the number of observed ASVs between groups, the Kruskal-Wallis test was utilised to address the diversity variations related to dpi and different treatments. To visually represent microbial composition variation and gain insights into patterns within the microbial community, PCoA was used. To test for group differences in microbial composition, permutational multivariate analysis of variance (PERMANOVA) was applied in a detailed exploration of the dataset (Anderson, 2001). Analysis of composition of microbiomes (ANCOM) (Mandal *et al*., 2015) was employed at phylum, class, order, family and genus level to identify bacterial taxa exhibiting differential abundance between groups and to detect whether specific taxa were associated with treatments.

In the AE study (Paper IV), all statistical analysis was performed with R (R Core Team, 2019). For *C. jejuni* plate counting data, Quasi-Poisson regression was used for significance analyses, and Tukey HSD was used for multiple pairwise comparisons. Quasi-Poisson regression was used instead of Poisson regression, which assumes that the mean and variance are equal, since the plating data exhibited overdispersion (variance was greater than the mean). For sequencing data (caecal samples), mixed effects linear models were fitted and analysed using the R packages lme4, lmerTest, pbkrtest and emmeans. These packages provide tools for fitting mixed effects models, conducting hypothesis tests and estimating marginal means. In the models, age, feed treatment and *C. jejuni* strain were used as fixed effects, and pen as a random effect.

In all papers, the probability value denoting statistical significance was set at $p \leq 0.05$.

5. Main Results

The main results obtained in the experiments presented in Papers I-IV are summarised below. For a complete description of the results, see the respective papers.

5.1 Paper I

Examination of the inhibitory effect of grass and maize silage extracts on *in vitro* growth of *C. jejuni* revealed inhibitory potential of both extracts, with grass silage demonstrating superior effects. Among the microbial strains tested as silage inoculum, *Lactiplantibacillus plantarum* 256 exhibited the most effective inhibitory potential. Heat-treated preparations provided a similar level of inhibition as untreated preparations, while neutralised preparations lost their inhibitory effect. Therefore, the primary mechanism of inhibition was concluded to be acid-dependent.

5.2 Paper II

Daily consumption of *L. plantarum* 256-inoculated water was found to increase BW during the starter period in R-308 birds. Adverse effects of haylage on BW and FI in R-308 birds were observed, while RR birds showed no significant adverse effects of forage inclusion.

5.2.1 Forage parameters

In monthly measurements during the experimental period, silage consistently exhibited higher LAB concentrations (8.0, 7.4, 7.2 log CFU/g) than haylage (5.0, 3.8, 3.0 log CFU/g), with a gradual decline over time. Silage also displayed lower pH (4.4) than haylage (6.2), when measured prior to the trial.

5.2.2 Treatment impacts on production performance

The R-308 birds subjected to treatment with LPW exhibited the highest BW in the initial period of the experiment (7 and 14 days of age), but this effect was no longer apparent at the end of the study (42 days) (Paper II). Silage inclusion in the diet caused a reduction in BW at days 28 and 42 compared with the control group, whereas feed consumption was not compromised. Haylage inclusion in the diet adversely affected BW and FI throughout the entire experiment compared with all other groups. Additionally, the lowest water intake was observed in the haylage group. In RR birds, there were no significant differences in BW, FI and water intake between the dietary treatments. However, birds in the LPW treatment showed a tendency for the highest BW at 7 days of age (Paper II). No significant differences in mortality were observed in either hybrid. In both hybrids, numerically higher consumption of silage than haylage was observed (Table 3).

Table 3. Daily forage consumption by Ross 308 and Rowan Ranger hybrids in *L. plantarum* 256 study

	Ross 308		Rowan Ranger	
Type of forage	Silage	Haylage	Silage	Haylage
Mean consumption (g)	86	58	67	50
% of feed intake	14	11	10	8

5.2.3 Impact of forage on gizzard condition

The inclusion of forages in the diet of both R-308 and RR birds resulted in a numerical increase in relative weight of the gizzard with contents compared with control birds fed only pellets. The different dietary treatments had no significant impact on broiler gizzard surfaces.

5.3 Paper III

Efficacy of *L. plantarum* 256 in reducing *C. jejuni* colonisation was not conclusively demonstrated in either of the broiler types during the rearing period. The treatments did not induce any significant alterations in the caecal microbiota. However, proportional changes in the bacterial composition following the *C. jejuni* challenge were observed, indicating a colonisation effect.

5.3.1 *Campylobacter jejuni* colonisation

Prior to the *C. jejuni* challenge, all birds tested negative for *C. jejuni*. Direct administration of *L. plantarum* 256 through drinking water (10^7 CFU/mL) did not have a significant impact on *C. jejuni* loads in either hybrid R-308 or RR broilers. In R-308 birds, the silage treatment gave an initial reduction in *C. jejuni* load (2.01 log) at 1 dpi as determined by culture, but this effect did not persist until the end of the experiment (19 dpi). In RR birds, no significant treatment effects on *C. jejuni* colonisation were found. Analysis of caecal samples by qPCR revealed no significant influence of dietary treatments on *C. jejuni* loads in either hybrid.

5.3.2 Changes in caecal microbiota

Sequencing analysis revealed no treatment effects on caecal microbiota in the gut of broilers. However, proportional changes in bacterial composition after the *C. jejuni* challenge were observed. There were clear trends in relative abundance of various genera, but also large individual variations in caecal microbiota composition among birds within the same treatment group at each sampling point.

At phylum level, *Firmicutes* and *Bacteroidota* dominated the caecal microbiota of both R-308 and RR birds, comprising 97% and 92%, respectively, of bacterial relative abundance at all sampling points across all treatment groups. In R-308 birds, a decrease in relative abundance of *Firmicutes* at 3 dpi (peak of *C. jejuni* colonisation) was subsequently compensated for by a significant increase in *Bacteroidota*. In RR birds, the reverse pattern was observed (for detailed description, see Paper III).

Among the notable observations at genus level in R-308 birds, *Bacteroides* dominated pre- (-1) and post- (3 dpi) challenge, but its abundance declined significantly by 20 dpi to the advantage of other genera (Table 4). *Clostridia* UCG-014 steadily increased in abundance, becoming the most abundant genus at 20 dpi. Relative abundance of *Lactobacillus* decreased post-challenge, followed by recovery to higher levels than pre-challenge by 20 dpi. *Clostridia* vadinBB60 abundance declined at 3 dpi and remained at similar level at 20 dpi. *Faecalibacterium* and *Escherichia-Shigella* abundance decreased post-challenge, but increased again by 20 dpi.

Notable changes were observed also in RR hybrids, where *Faecalibacterium* was the second most abundant genus pre-challenge and became the most abundant genus post-challenge (3 and 13 dpi), exceeding

Bacteroides which dominated the caecal microbiota initially. Relative abundance of *Bacteroides* declined considerably after the challenge but it remained in second position, while that of *Clostridia* UCG-014 peaked at 3 dpi, followed by a moderate decrease post-challenge. *Lactobacillus* abundance increased consistently throughout the sampling period, while *Clostridia* vadinBB60 showed a continuous decrease. Relative abundance of *Escherichia-Shigella* decreased post-challenge and remained at similar level at 13 dpi.

Table 4. The mean relative abundance (%) of genera with notable changes in the gut microbiota of broiler hybrids observed in both microbiota studies

	Lactiplantibacillus plantarum 256 study						Algal extract study	
Hybrid	R-308	R-308	R-308	RR	RR	RR	R-308	R308
Challenge status	BC	AC	AC	BC	AC	AC	BC	AC
Age of birds (days)	21	25	42	28	32	42	14	37
Days post-infection	-1	3	20	-1	3	13	-3	20
Genus	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Bacteroides	14.5	23.7	13.4	25.9	14.6	18.5	0.01	0.2
Faecalibacterium	9.4	8.2	13.7	14.1	14.7	25.9	0.1	26.3
Clostridia UCG-014	4.1	9.9	16.1	6.9	13.9	9.5	6.6	0.6
Clostridia vadinBB60	12.7	6.8	6.4	5.3	5.0	3.3	0.1	2.8
Lactobacillus	1.7	0.9	2.9	1.1	2.0	2.6	2.4	0.3
Escherichia-Shigella	1.2	0.7	1.1	2.8	1.3	1.3	11.5	1.9

* R-308 = Ross 308; RR = Rowan Ranger; BC = before challenge; AC = after challenge

5.4 Paper IV

5.4.1 *Campylobacter jejuni* colonisation

All birds in Paper IV tested negative for *C. jejuni* prior to the challenge and successful colonisation with both *C. jejuni* strains was confirmed after the challenge. Feed supplementation with AE did not significantly affect *C. jejuni* colonisation in R-308 birds, with agar plate counts revealing no notable differences between birds on the AE diet and the basal diet at any of

the sampling points. No differences were found in the ability of the two *C. jejuni* to colonise the gut of broiler chickens, as similar *C. jejuni* levels in the gut from day 1 to day 19 were observed in all treatment groups. Rapid *C. jejuni* colonisation peaked three days post-challenge, followed by a sustained level until the end of the experiment (Paper IV).

5.4.2 Changes in caecal microbiota

No significant effect of AE supplementation on development of the caecal microbiota was observed. Comparison of caecum microbial composition at 14 days of age (pre-challenge) and 37 days of age (post-challenge) revealed distinct changes in the microbiota post-challenge. In addition, considerable individual variation in caecal microbiota among the birds was observed.

Genus-level bacterial shifts were observed (Table 4), characterised by a pronounced decrease in relative abundance of *Escherichia-Shigella* after the challenge. In contrast, *Faecalibacterium*, a minimally abundant genus pre-challenge, increased substantially in abundance after inoculation of the birds with *C. jejuni*. Concurrently, there were noticeable declines in abundance of the genera *Clostridia* UCG-014 and *Lactobacillus* post-challenge.

6. General Discussion

The studies on which this thesis is based (Papers I-IV) were carried out to investigate whether alternative diets and dietary supplements (containing LAB or laminarin-rich AE) can alter the gut microbiota in broilers and provide a more resilient gut environment, and thereby lead to a reduction in *C. jejuni* load in broiler caeca and faeces. This chapter discusses the key findings of these studies in the context of relevant scientific literature. The scientific contributions of the thesis and future implications of the work are reported in Chapters 7 and 8, respectively.

6.1 Lactic acid bacteria

Before investigations into the potential of LAB in reducing *C. jejuni* in broiler caeca, *in vitro* testing was performed (Paper I). Of the three LAB strains tested (*L. plantarum* 256, *L. plantarum* E-78076, and *L. reuteri* DSM 20016), silage extract containing *L. plantarum* 256 exhibited the most effective *C. jejuni* inhibitory potential. This strain was originally isolated from silage (Johansson *et al.*, 1995), fitting with the definition of *L. plantarum* as a highly adaptable lactobacillus strain prevalent in various fermented products (Rivas *et al.*, 2006).

It has been suggested that production of antimicrobial metabolites by *L. plantarum* and *L. reuteri* could mediate growth inhibition of Proteobacteria, including *C. jejuni* (Asare *et al.*, 2020). The inhibition capacity observed for heat-treated silage extracts in Paper I, which was comparable to that in untreated extracts, indicates that the mechanism of action was not dependent on proteinaceous compounds. Subsequent abolition of the growth inhibition effect through pH neutralisation of the silage extracts led to the conclusion that the primary mechanism of action was acid-dependent. This observation

is in agreement with results reported by Tomusiak-Plebanek *et al*. (2022), who demonstrated with a well diffusion method that reduced pH (attributed to lactic acid) plays a key role in inhibiting the growth of *Campylobacter* bacteria *in vitro*.

Lactiplantibacillus plantarum 256 was identified as the most promising strain in the *in vitro* study (Paper I), and was selected for further investigation as an inoculant of grass material for silage. Concurrently, haylage as a non-inoculated forage similar to silage was produced. Inclusion of haylage treatment in the study design served the purpose of distinguishing whether a potential significant effect on *C. jejuni* load in broilers resulted from the selected *L. plantarum 256* strain or from the diverse composition of LAB present in the forages.

Forage inoculation with LAB at ensiling is a well-established practice, recognised to promote lactic acid fermentation, suppress the proliferation of undesirable microorganisms and enhance preservation (Cai *et al.*, 1999). Expected superior lactic acid fermentation in the grass inoculated with *L. plantarum* 256 (10^8 CFU/g fresh matter) in comparison with untreated grass was confirmed by observed higher LAB concentrations and lower pH in the silage treatment compared with the haylage treatment (Papers II and III). The elevated LAB concentrations in silage suggest metabolism of sugars, leading to production of a greater amount of organic acids than in haylage. However, these parameters do not necessarily mean that the quality of haylage provided to the birds was compromised. For example, Müller (2005) concluded that from a nutritional point of view, higher pH and reduced organic acid levels in haylage should not be interpreted as signs of inadequate preservation, since several other parameters (such as ammonia levels, butyric acid, fungal counts) are of equal importance. However, since the main aim with forage inclusion was to test its potential to reduce *C. jejuni* colonisation in broilers, these parameters were not recorded in this thesis.

Both silage and haylage were produced using routine farming practices, to demonstrate their potential applicability for use in organic broiler production settings, so unsterilised forages were used in preparation of silage and haylage bales. With this set-up, epiphytic LAB naturally present in the forage (Webster, 2002) contributed to the ensiling process, explaining the relatively high counts of LAB observed in the haylage, despite the absence of inoculation in its preparation.

6.2 Broiler performance

To assess whether any potential reduction in *C. jejuni* within the broiler gut was caused by the complex LAB present in the forage or by the *L. plantarum* 256 itself, direct provision of this strain via drinking water was included as a treatment. The impact of LPW on broiler performance was also investigated (Paper II). Although feed supplementation remains the most common method of probiotic administration in poultry production, provision via drinking water is suggested to be more effective in enhancing production performance (Karimi Torshizi *et al.*, 2010). The temporary positive impact of providing *L. plantarum 256* in water on the initial BW of R-308 birds and the tendency for increased initial BW in RR birds (Paper II) contradicts findings by Peng *et al.* (2016). In their study, diet supplementation with 2×10^9 CFU/kg *L. plantarum* B1 significantly influenced BW in the second half of the trial (22-42 days of age), while no effect was observed in the first half of the trial (1-21 days). In a study by Karimi Torshizi *et al.* (2010), a multi-strain probiotic supplement (including *L. plantarum*) administered via drinking water to R-308 broilers was found to give an increase in BW during the starter period compared with the control group, aligning with observations in Paper II. However, probiotic supplementation had a positive impact on BW throughout their entire study, while also improving FI and FCR (Karimi Torshizi *et al.*, 2010). It is important to highlight that direct comparison of results from probiotic studies is rarely straightforward, primarily due to variations in strain selection, dosage levels and administration methods employed.

The effect of dietary fibre on broiler performance has been shown to depend on many factors, such as fibre particle size (Amerah *et al.*, 2009), inclusion level (Jiménez-Moreno *et al.*, 2009) and dietary energy level (Tejeda & Kim, 2021). In this thesis, silage provision to R-308 birds reduced BW at d 28 and 42, while FI was not compromised. This finding may be related to the energy-diluting effect of forage intake (Latshaw, 2008). Interestingly, Ranjitkar and Engberg (2016), who studied R-308 broilers fed maize-based diet with 15% inclusion of crimped kernel maize silage (CKMS) on a DM basis, fed as total mixed ration, found that the birds displayed comparable BW and FI to a maize-only control group. This discrepancy in results may be explained by the four-fold higher content of metabolisable energy in CKMS than in the energy-poor grass silage used in Paper II.

Throughout the experiment in Paper II, inclusion of haylage adversely affected FI and BW, suggesting that haylage led to reduced voluntary feed consumption and subsequently impacted growth performance. A potential cause of this could be fast-growing broiler sensitivity to dietary quality (Tufarelli *et al.*, 2018), where the drier texture of haylage (attributed to higher DM content) likely diminished the birds' interest in the feed. As a probable consequence, water intake was lowest in haylage-fed group, which may reflect the correlation between feed consumption and water intake in poultry (Aggrey *et al*., 2023).

Daily consumption of forage and supplementation with *L. plantarum* 256 did not give any significant differences in FI, BW and water intake in RR birds, indicating potential to mix forage into the pelleted diet of slower-growing hybrids at an inclusion level of 15% without negatively impacting their performance. It is well established that fast-growing broilers tend to have higher FI than slower-growing hybrids, and the numerically lower intake of forage in RR birds, in contrast to R-308 birds, may have contributed to these findings.

Previous studies investigating inclusion of forages in broiler diets are limited. In both hybrids, there was a preference for silage over haylage, with consumption reaching 14% of the feed intake in R-308 and 10% in RR, compared with the 15% initially provided in the diet. The lower interest of the birds in consuming the forage provided could be attributable to potential unpalatability or to the presence of unpleasant post-ingestion effects (Gillette *et al.*, 1983), although this remains speculative. Interestingly, alfalfa is regularly offered as an environmental enrichment in commercial broiler production in Norway and birds are observed to prefer playing with alfalfa than eating it. Analysis by Kittelsen *et al.* (2023) revealed that the birds predominantly consumed the leaf fraction of alfalfa roughage, while leaving the stipe untouched. Therefore, the nutritional contribution of the included forage was concluded to be minimal.

The gizzard is a specialised stomach constructed of thick muscular walls, with thick and thin muscles that are asymmetrically arranged, resulting in both a rotary movement and a crushing movement when the gizzard contracts (Svihus, 2011). In both hybrids studied in Paper II, numerically higher relative weight of the gizzard was observed in forage-fed birds compared with birds fed only pellets, likely due to the high content of fibre in the forages. It has been shown that fibre-rich diets increase digesta retention time

and prolong the grinding phase in the gizzard, with the increased grinding activity associated with development of the gizzard (Svihus, 2011).

6.3 *Campylobacter jejuni* colonisation

It is common practice to perform culture of faeces samples or cloacal swabs before experimental *C. jejuni* inoculation (Flaujac Lafontaine *et al.*, 2019; Paul *et al.*, 2014; Connell *et al.*, 2012), in order to confirm that all birds are *C. jejuni*-negative prior to challenge. This was verified in Papers II, III and IV in this thesis, with successful *C. jejuni* colonisation achieved by both inoculation methods employed. In addition, rapid *C. jejuni* colonisation peaking three days post-challenge was observed in both studies, in agreement with previous findings (Shanker *et al.*, 1990).

Addition of probiotic to the water has been identified as a promising feed-based measure to enhance gut microbial maturation and diversity, potentially reducing the prevalence of resistant bacteria in broilers (Soumeh *et al.*, 2021). However, in the *L. plantarum* 256 study in Paper III, direct administration of LPW (10^7 CFU/mL) did not have a significant impact on *C. jejuni* loads in R-308 or RR birds as determined by culture. Interestingly, Ghareeb *et al.* (2012) observed a significant reduction in *C. jejuni* caecal colonisation in R-308 birds when a daily dose of multi-strain probiotics was administered via water (2 mg/bird per day), decreasing from 7.85 log CFU/g to 2 log CFU/g at 15 days post-challenge. Furthermore, Smialek *et al.* (2018) found that feed supplemented with 10^7 CFU/g of a multispecies probiotic (including *L. plantarum*) reduced the *Campylobacter* spp. population in broiler caeca prior to slaughter in a commercial farm setting. One plausible explanation for the divergence in findings could be that multi-strain probiotics are reported to have higher efficacy than single strains, a difference probably linked to synergistic interactions between strains (Chapman *et al.*, 2011). Furthermore, while the *in vitro* study in Paper I in this thesis revealed an inhibitory impact of *L. plantarum* 256 on *C. jejuni* growth, this effect was not evident in subsequent animal experiments in Papers II and III. This discrepancy can be related to findings by Smialek *et al.* (2018) that *in vitro* studies do not take into account the variability and complexity of the birds' GIT environment and their interaction with probiotics and *Campylobacter* strains. In line with this, a study by Arsi *et al.* (2015) found that among 26 LAB isolates showing significant *in vitro*

inhibitory activity, only three exhibited a notable reduction (approximately 1-2 log) in *Campylobacter* counts during a broiler trial.

There have been few *in vivo* studies to date exploring the potential of silages to reduce the *C. jejuni* load in broilers. As mentioned, fast-growing birds are known to have higher FI than slow-growing hybrids (Quentin *et al*., 2004; Sarica *et al*., 2020), making it interesting to investigate the impact of forages on *C. jejuni* reduction in both fast-growing and slower-growing broilers in this thesis. In R-308 birds, the silage treatment gave an initial reduction in *C. jejuni* load (2.01 log) at 1 dpi, as determined by culture (Paper III), but this effect did not last. It can be speculated that the anticipated higher intake of silage in this fast-growing broiler hybrid may exert an inhibitory effect against low loads of *C. jejuni* ingestion, but is not capable of providing protection against *C. jejuni* colonisation *per se*. Additionally, when expressed as CFU/g, the reduction in *C. jejuni* load in one group may be artificially biased compared with the other groups. In RR birds, no *C. jejuni*-reducing effects of silage consumption in the initial part of the broilers' life was noted. Similarly, Ranjitkar and Engberg (2016) observed no significant influence on intestinal colonisation by *C. jejuni* in R-308 broilers following CKMS inclusion (15% and 30% on DM basis) in a maize-based diet. These findings may be explained by insufficient amounts of silage consumption in both studies, preventing expression of a *Campylobacter*-reducing effect by lowering the pH in the GIT or inducing changes in gut microbiota composition.

In RR birds, no significant effect of treatments on *C. jejuni* colonisation was seen in analysis of faecal samples by culture and caecal samples by qPCR. Similarly, qPCR analysis of caecal samples from R-308 birds did not show any treatment effects (Paper III). Taken together, these findings indicate that interventions involving silage, haylage and daily provision of LPW were ineffective in reducing *C. jejuni* colonisation in both R-308 and RR hybrids.

Laminarin-rich extract from *Laminaria* spp. has previously shown promising effects in modulation of the microbial profile of broilers (Venardou *et al.*, 2021) and an increase in interleukin 17A expression in the broiler duodenum, which is known for its role in the immune response against several infectious agents, including *Campylobacter* (Connerton *et al.*, 2018). However, in the study in Paper IV, laminarin-rich AE supplementation (290 ppm) of feed did not significantly affect *C. jejuni*

colonisation in R-308 broilers, as agar plate counts revealed no great differences between birds on the AE diet and birds on the basal diet. This aligns with findings by Sweeney and O'Doherty (2016) of no significant differences in *C. jejuni* colonisation in the initial post-hatch period in broilers receiving 250 ppm of laminarin in the basal diet. Taken together, the findings to date indicate that laminarin-rich algal extracts have a limited direct antimicrobial impact in terms of reducing *C. jejuni* colonisation in broiler caeca.

6.4 Changes in caecal microbiota

A study by Oakley *et al.* (2014) highlighted the important role played by the gastrointestinal microbiota in broiler nutrition and investigations in this area are still ongoing. However, it should be noted that despite the advanced microbiota investigation methods available today, the findings from microbiota analysis related to dietary interventions should be interpreted with great caution (Rychlík, 2020).

Sequencing analysis of caecal samples from the birds in different treatments in this thesis revealed no treatment effects of silage, haylage and *L. plantarum* 256 (Paper III) or AE (Paper IV) on development and composition of the microbiota. However, proportional changes in bacterial composition occurred after the *C. jejuni* challenge in both studies, suggesting a potential impact of *C. jejuni* colonisation on gut microbiota composition (Table 4). It should be noted that the main aim of the animal studies in this thesis was to investigate whether daily provision of *L. plantarum* 256 (Paper III) or AE (Paper IV) can have a direct effect on broiler resistance to *C. jejuni* colonisation and whether any effect arising could possibly be explained by their caecal microbiota composition, in contrast to birds fed a control feed. Therefore, *C. jejuni* non-challenged control groups were not included in the study design. This limitation made it impossible to draw direct conclusions about whether changes in caecal microbiota composition observed after the challenges were linked to *C. jejuni* colonisation or to the age of the birds. Age is known to be is a significant influencing factor in the caecal microbiota of broilers (Ocejo *et al.*, 2019), and should be considered in interpretation of results from feeding trials. In addition, while trends in relative abundance of various genera were observed post-challenge, there was great individual variation in microbiota composition among birds within the same treatment

group at each sampling point. Similarly, Stanley *et al.* (2013) observed significant individual variation in broiler caecal microbiota within uniformly derived and treated groups under strictly controlled experimental conditions. Those authors attributed this variation to limited exposure to maternally acquired bacteria and subsequent environmental bacterial colonisation of newly hatched chickens. Several previous studies have explored specific differences in the composition of intestinal microbiota associated with the presence of *C. jejuni* in the broiler gut, but the majority of identified taxa have been classified only at order or family level, lacking genus-level differences (Chintoan-Uta *et al.*, 2020; Connerton *et al.*, 2018). Despite the previously mentioned experimental limitations, this thesis investigated whether there are some specific associations between *Campylobacter* colonisation and caecal microbiota composition in broilers on genus level (Table 4).

6.4.1 Microbial shifts after *Campylobacter jejuni* challenge

One of the notable observations on genus level in the *L. plantarum* 256 study in this thesis was that caecal microbiota composition was dominated by *Bacteroides* and *Faecalibacterium* in both hybrids studied. However, different pattern of changes in relative abundance of these genera in the two hybrids were observed after the *C. jejuni* challenge (Table 4). A significant increase in *Bacteroides* was observed in R-308 birds, while in RR birds *Faecalibacterium*, the second most abundant genus pre-challenge, became the most abundant genus post-challenge. These observations are in general agreement with findings by Pang *et al.* (2023) that the most abundant genera in *Campylobacter*-positive birds are *Bacteroides*, followed by *Phascolarctobacterium* and *Faecalibacterium*. Interestingly, the genus *Phascolarctobacterium*, which was not detected in the analyses in this thesis, is reported to be involved in short-chain fatty acid (SCFA) production and is a potential candidate for reducing *Campylobacter* through competitive exclusion (McKenna *et al.*, 2020). The converse pattern of *Bacteroides* and *Faecalibacterium* abundance observed in R-308 and RR in this thesis may be attributable to breed differences. It has previously been found that the microbiota composition of three different broiler hybrids (Ross, Cobb, Hubbard), all obtained from a single commercial hatchery and co-housed in a biosecure housing unit, exhibit variations (Richards *et al.*, 2019).

Interestingly, the AE study revealed an increase in relative abundance of both *Bacteroides* and *Faecalibacterium* following the *C. jejuni* challenge (Paper IV). However, while *Faecalibacterium* exhibited a notable post-challenge increase and emerged as the most abundant genera in broiler caeca, *Bacteroides* was present in only minor relative abundance in caeca throughout the study. It is important to note, however, that interpretation of pre- and post-challenge observations in the AE study is highly speculative due to the considerable age difference between pre- and post-challenge samplings, specifically 23 days. In contrast, in the *L. plantarum* 256 study the pre- and post-challenge samplings were conducted only four days apart. Therefore, the age effect in the AE study likely exerted a more pronounced influence on gut microbiota than the potential impact of *C. jejuni* presence. Nevertheless, the results from that study are included here to provide a general overview of microbiota development in the microbiota studies performed in this thesis.

Relative abundance of *Escherichia-Shigella* decreased in both microbiota studies, while a concurrent rise in abundance of *Clostridia UCG-014* was observed after the *C. jejuni* challenge in the *L. plantarum* 256 study. Awad *et al.* (2016) observed similar changes in two-week-old R-308 broilers challenged with 1×10^8 CFU of *C. jejuni* (NCTC 12744). In that study, the *Campylobacter*-positive birds showed a significant decrease in *Escherichia coli* abundance and an increase in *Clostridium spp*. compared with *Campylobacter*-negative birds. However, it is important to note that the higher abundance of *Clostridia* UCG-014 observed in the *L. plantarum* 256 study was accompanied by a simultaneous decrease in the relative abundance of the genus *Clostridia* vadinBB60. Moreover, opposite shifts in the relative abundance of *Clostridia* UCG-014 and *Clostridia* vadinBB60 were observed in the AE study at dpi 20 (Table 4), likely linked to the age effect on caecal microbiota development. These observations highlight the complexity of avian gut microbial communities.

Objective in the *L. plantarum* 256 study was to assess LAB administration approaches in terms of enhancing LAB presence in broiler GIT, in order to create an inhospitable environment for *Campylobacter*. The results showed that silage LAB inclusion or *L. plantarum* 256 supplementation in water did not significantly affect relative abundance of *Lactobacillus* in broiler caeca compared with the control. A possible impact of *C. jejuni* colonisation on *Lactobacillus* abundance was noted, as relative

abundance of *Lactobacillus* decreased in R-308 birds in both microbiota studies after the *C. jejuni* challenge at dpi 3 in the *L. plantarum* 256 study and at dpi 20 in the AE study, irrespective of treatment (Papers III and IV). A connection between the genera *Lactobacillus* and *Campylobacter* has previously been observed by Sofka *et al.* (2015), who found that LAB counts were significantly higher in *Campylobacter*-negative samples from broiler flocks compared with *Campylobacter*-positive samples. However, this correlation was not noted in RR birds in this thesis, as relative abundance showed a continual rise over time post-challenge, independent of treatment. Furthermore, although the genus *Lactobacillus* exhibited relatively high abundance in RR caeca in the silage and LPW groups compared with the control (not statistically significant) pre-challenge, its presence did not impact *Campylobacter* loads after the challenge.

7. Conclusions

The main finding in this thesis was that presence of *C. jejuni*, age and broiler type (fast- or slower-growing hybrid) had greater effects on caecal microbiota composition than the different feed additives tested (*L. plantarum* 256 and laminarin-rich algal extract). These additives did not alter caecal microbiota composition and did not prove to be effective in reducing the load of *C. jejuni* in the gut of broiler chickens. Inclusion of silage in the diet initially gave a reduction in *C. jejuni* load in R-308 birds, but this effect was not sustained. In addition to the main conclusions outlined above, other notable findings of the experiments were as follows:

- *L. plantarum* 256 displayed promising inhibition of *C. jejuni* growth *in vitro*. However, this effect was not confirmed in a subsequent *in vivo* experiment.
- Daily water supplementation with *L. plantarum* 256 showed potential to increase broiler weight at the beginning of the rearing period, but this effect did not persist.
- Higher feed intake of silage than of haylage was observed in both fast-growing and slower-growing hybrids.
- In the Ross 308 hybrid, haylage had an adverse effect on production performance throughout the experiment, while an adverse effect of silage was observed only at days 28 and 42.
- No adverse effect of forages on broiler production performance was observed in the Rowan Ranger hybrid. This suggests that forage can be included at 15% (DM basis) in pelleted feed for Rowan Ranger broilers without negatively affecting performance.

8. Practical Implications and Future Perspectives

Although there was no overall effect of the dietary interventions tested in this thesis in terms of *C. jejuni* reduction in the broiler gut, initial inhibition of *C. jejuni* upon inclusion of silage in the feed was observed. This suggests that further investigations may be of interest. Optimisation measures could include changing from grass-based to wheat-based silage, as cereal-based fermented feed is likely to be more palatable to birds, potentially leading to increased consumption of experimental feed. This higher consumption may foster greater LAB gut colonisation and upper gut acidification, resulting in a more pronounced inhibitory effect on *C. jejuni*. Outside the framework of this thesis, additional pilot testing of wheat ensiled with 10 different lactobacilli strains was conducted. Notable growth inhibition was observed *in vitro*, particularly with the strain *Lactobacillus panis* DAF355. Further research, including feeding trials evaluating wheat silage inclusion levels and their impact on broiler performance, is necessary to determine whether the *in vitro* inhibitory effect translates into a substantial reduction in *C. jejuni* in *in vivo*. However, even if it proves to be effective, the practicality of this intervention in broiler production is debatable due to the increased labour required. Broiler farmers, especially in countries like Sweden with moderate and low levels of *Campylobacter* prevalence in their flocks, may be hesitant to invest time and resources in such additional work.

Scandinavian commercial production demonstrates that strict biosecurity standards can greatly decrease the prevalence of *Campylobacter*-positive flocks. However, implementing this seemingly straightforward measure still appears to pose challenges in many countries.

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Popular science summary

Birds, including broiler chickens, are the closest living relatives of dinosaurs. However, broilers have undergone dramatic transformations over time. Through genetic selection and optimised feeding strategies, current commercially raised broilers achieve rapid growth, from an average of 40 g at hatching to a substantial 2.3 kg within just 35 days. Hatching in hatcheries means that the chicks do not come into contact with the mother hen or with the environment in which the hen lives. Hatching in a cleaner environment enables higher biosecurity, which protects the chick from infectious agents. However, it does not offer the same gradual establishment of microorganisms in the digestive tract that occurs when the chick is hatched naturally under a brooding hen.

In the outdoor environment, there are many different microorganisms and these can be unintentionally brought into the broiler barn, via staff, visitors or equipment. For example, it is common to use a machine that collects broiler chickens for slaughter with the help of rotating rubber fingers. When the same machine is used on several farms, this poses a potential risk of unwanted microorganisms being transferred between farms, even if the machine is cleaned after each use. One microbial risk is the bacterium *Campylobacter jejuni*. Many wild bird species naturally carry *C. jejuni* in their gastrointestinal tract, and rodents and insects can spread the bacteria in the farm environment. In international comparisons, the proportion of broiler flocks that carry the bacteria at slaughter is low in Swedish commercial chicken farming, but the proportion usually increases during the summer months, especially in organic production. Birds that are raised under organic conditions must have daily access to roughage and the opportunity to stay outdoors. The stay outdoors increases the likelihood of the broilers coming into contact with, and becoming infected by, *C. jejuni* as they explore their

environment and perform foraging behaviours. Therefore, it is likely that outdoor stays increase the risk of *C. jejuni* establishing itself in the chicken's intestine and spreading to other individuals in the flock.

Although *C. jejuni*-positive flocks typically show no symptoms, the bacterium can cause infections of the gastrointestinal tract in humans, resulting in severe diarrhoea, fever and vomiting. In fact, gastrointestinal infection with *C. jejuni* is the most frequently reported disease transferred from animals to humans within the European Union. The main reason behind infection in humans is use of inappropriate kitchen routines when handling *C. jejuni*-contaminated chicken meat, such as not keeping raw meat separate from other foods or insufficient heating during cooking.

This thesis investigated the possibility of making it difficult for *C. jejuni* to establish itself in the chicken intestine by using various feed supplements in the broiler diet. Fermented feed rich in lactic acid bacteria and feed with added algae extract were evaluated, since both have been identified as promising additives to influence the composition of microbes in the gastrointestinal tract of chickens in a way that could reduce the occurrence of *C. jejuni*. Studies on these additives were conducted in a controlled environment where all chickens were intentionally infected with *C. jejuni*.

In one study, the effect of daily intake of the lactic acid bacterium *Lactiplantibacillus plantarum* 256 (LP256) was investigated. The chickens ingested the bacterium either via water or via silage, where the bacterium was added to cut grass being wrapped in plastic for fermentation. The comparison also included hay silage (haylage), which consisted of the same grass but slightly dried before storage in plastic bales and with no added LP256. In another study, algal extract made from the brown algae *Saccharina latissima* was evaluated as a feed supplement to strengthen the intestinal bacterial flora of chickens and possibly inhibit the establishment of *Campylobacter*. The effect of the feed supplements was evaluated by measuring the establishment of *Campylobacter* in chickens with or without feed supplements and assessing how the microbial composition in the caecum of the chickens developed. Effects on chicken body weight and feed intake were also evaluated.

Daily intake of LP256 and algae extract did not effectively reduce the establishment of *C. jejuni* in the intestine of broiler chickens. The chickens that received the LP256 supplement via their drinking water gained more weight than the other chickens at the beginning of life. Silage and haylage

had a better effect when given to chickens of a slower-growing breed, while silage reduced feed intake and growth in some cases and haylage showed negative effects throughout the study in broilers of a faster-growing breed. There are several possible reasons why the feed supplements tested did not inhibit establishment of *C. jejuni* in the gut of commercial broiler chickens. In future studies, the effect of other bacterial species and other ways of supplying the bacteria to broilers, for example via different types of feed, should be evaluated. The positive effect of supplying lactobacilli of the strain LP256 via drinking water on the early weight development of broilers is interesting and more research is needed to understand the underlying reasons.

Populärvetenskaplig sammanfattning

Alla fåglar, inklusive slaktkycklingar, är de närmaste levande släktingarna till dinosaurier. Trots denna uråldriga koppling har slaktkycklingen förändrats dramatiskt över tiden. Genetiskt urval och optimerade utfodringsstrategier har resulterat i att kommersiellt uppfödda slaktkycklingar med en genomsnittlig vikt på 40 gram vid kläckning, väger 2300 gram inom bara 35 dagar. Kläckning på kläckerier innebär att kycklingen varken kommer i kontakt med mammahönan eller med den miljö hönan lever i. Kläckningen i en renare miljö innebär en högre biosäkerhet vilket skyddar kycklingen från smittämnen, men ger inte samma gradvisa etablering av mikroorganismer i matsmältningskanalen som uppkommer när kycklingen kläcks fram under en ruvande höna.

I vår utomhusmiljö finns många olika mikroorganismer och dessa kan oavsiktligt föras in i kycklingstallet till exempel via personal, besökare eller utrustning. Det är till exempel vanligt att använda en maskin som med hjälp av roterande gummifingrar samlar in kycklingarna inför slakt. Då samma maskin används på flera gårdar utgör den en potentiell risk för att oönskade mikroorganismer överförs mellan gårdarna, även om den rengörs efter varje användning. En mikrobiell risk är bakterien *Campylobacter jejuni* (C. *jejuni*) sommånga vildfågelarter bär naturligt i mag- tarmkanalen. Gnagare och insekter kan sprida bakterien i gårdsmiljön. I internationell jämförelse är andelen slaktkycklingflockar som bär på bakterien vid slakt låg i svensk kommersiell kycklinguppfödning, men andelen ökar vanligtvis under sommarmånaderna och särskilt i ekologisk produktion. Fåglar som föds upp under ekologiska förhållanden ska ha daglig tillgång till grovfoder och möjlighet att vistas utomhus. Vistelsen utomhus ökar sannolikheten för att kycklingen ska komma i kontakt med och infekteras av *C. jejuni*, när den utforskar sin miljö och utför födosöksbeteenden. Därmed är det sannolikt att

utomhusvistelsen ökar risken för att *C. jejuni* etablerar sig kycklingens tarm och sprids till andra individer i flocken

Även om kycklingarna i *C. jejuni*-positiva flockar vanligtvis inte uppvisar några symtom kan bakterien orsaka infektioner i mag-tarmkanalen hos människor, med svår diarré, feber och kräkningar som följd. Faktum är att mag-tarminfektion med *C. jejuni* är den vanligaste av alla sjukdomar som överförs från djur till människa inom Europeiska unionen. Det är i samband med slakt som tarmbakterien hamnar på slaktkroppen och bristande köksrutiner vid hantering av kycklingkött, till exempel att det råa köttet inte hålls isär från andra livsmedel eller otillräcklig upphettning vid tillagning är de främsta orsakerna bakom smitta till människa.

I denna avhandling undersöktes möjligheten att via olika fodertillskott försvåra möjligheten för *C. jejuni* att etablera sig i kycklingtarmen. Fermenterat foder rikt på mjölksyrabakterier och foder med tillsats av algextrakt utvärderades i separata studier då båda alternativen identifierats som lovande strategier för att påverka sammansättningen av mikrober i kycklingars mag-tarmkanal på ett sätt som skulle kunna minska förekomsten av *C. jejuni*. Båda studierna genomfördes i kontrollerad miljö där alla kycklingar avsiktligt smittades med bakterien.

I den första studien undersöktes effekten av dagligt intag av mjölksyrabakterien *Lactiplantibacillus plantarum* 256 (LP256). Kycklingarna fick i sig bakterien antingen via vatten eller via ensilage där bakterien tillförts i samband med att gräset plastats in för att fermenteras. I jämförelsen ingick också hösilage, som utgjordes av samma gräs som torkats något innan lagring i plastad bal. Till hösilaget tillsattes inte LP256. I en efterföljande studie utvärderades algextrakt tillverkat av brunalgen *Saccharina latissima* som fodertillskott för att stärka tarmens bakterieflora hos kycklingar och eventuellt hämma etablering av *Campylobacter*. Effekten av fodertillskotten utvärderades genom att mäta etableringen av *Campylobacter* i kycklingar med eller utan fodertillskott samt hur den mikrobiella sammansättningen i kycklingarnas blindtarmar utvecklades. Även påverkan på kycklingarnas kroppsvikt och foderintag utvärderades.
Varken dagligt intag av LP256 eller algextrakt kunde effektivt reducera etableringen av *C. jejuni* i kycklingarnas tarmar. De kycklingar som fick tillskott av LP256 via sitt dricksvatten ökade mer i vikt än övriga kycklingar i början av livet. Dessutom visar resultaten att ensilage och hösilage fungerar bättre att ge till kycklingar av mer långsamtväxande ras medan ensilage

minskade foderintag och tillväxt i vissa fall och hösilage visade negativa effekter under hela studien hos kycklingar av mer snabbväxande ras. Det finns flera tänkbara orsaker till att fodertillskotten i våra studier inte kunde hämma etablering av *C. jejuni*. I framtida studier bör man utvärdera effekten av andra bakteriearter samt andra sätt att tillföra bakterierna till kycklingarna, tex via olika typer av foder. Den positiva effekten av att tillföra laktobaciller av stammen LP256 via dricksvattnet på slaktkycklingars tidiga viktutveckling är intressant, men mer forskning behövs för att förstå de bakomliggande orsakerna.

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"Tak končí – aspoň doufám - naše komédie. Zlo prohrává a dobro žije..."

II

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Silage and haylage as forage in slow and fast-growing broilers – effects on performance in *Campylobacter jejuni* infected birds

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ABSTRACT

1. This study investigated the effects of daily intake of silage or haylage on broiler production performance and organ development. Furthermore, effects of daily intake of *Lactobacillus plantarum* either *via* silage or by supplemented drinking water, on *Campylobacter jejuni* loads in faeces were studied.
2. To test this, a 42-d experiment using Ross 308 and a 63-d experiment with Rowan Rangers hybrids, were performed. Silage inoculated with *L. plantarum* strain 256 and haylage were fed in total mixed rations with mixtures of 85% of pellets and 15% of respective forage (DM-based weight). Feed intake (FI), forage intake, body weight (BW) and feed conversion ration (FCR) were monitored weekly. Mortality was recorded daily, and organ weights were registered at slaughter. Quantification of *C. jejuni* was performed by colony counts from faecal samples after culture on agar plates.
3. There was a negative effect of haylage on BW and FI in the fast-growing Ross 308 hybrid. Silage had a negative effect on BW only on week four and six. Water inoculated with *L. plantarum* 256 increased BW in the starter period. Interestingly, no significant adverse effect of forage inclusion was observed in the Rowan Ranger birds.
4. Relative weight of the emptied gizzard was higher in both Ross 308 and Rowan Ranger birds fed haylage and silage than in the control group. In Ross 308 birds, both forages significantly reased the relative weight of gizzard with digestive content when compared to birds fed solely pellets.
5. In both studies, higher consumption of silage than haylage was observed.
6. In conclusion, daily intake of *L. plantarum* 256 either *via* silage or supplemented in drinking water, was not effective in reducing the shedding of *C. jejuni* in either Ross 308 or Rowan Ranger hybrids at the end of the rearing period.

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Introduction

Forages (e.g. grass, clover), are feedstuffs containing a high amount of insoluble fibre, a group of plant compounds that cannot be broken down by digestive enzymes (Choct 2015). Commonly, insoluble fibre has been considered as a poultry diet diluent, causing adverse effects on feed intake and digestibility of the nutrients (Rougière and Carré 2010; Sklan et al. 2003). Despite this belief, recent publications have shown that moderate amount of insoluble fibre have a positive effect on nutrient availability (Svihus 2011), volume of gizzard contents (Hetland et al. 2003) and digestive traits, *e.g.* stimulated development of the upper digestive tract part (González-Alvarado et al. 2008). However, it has been shown that the effect of fibre on broiler performance depends on many factors, such as fibre particle size (Amerah et al. 2009) and inclusion level (Jiménez-Moreno et al. 2009).

Since the number of consumers demanding organically produced food is increasing, organic livestock farming is growing (Hughner et al. 2007). All organic birds in the European Union must have the possibility to range outdoors and have daily access to forage (Commission Regulation (EC) 889/2008). However, although the access to forage is required, guidelines for its quantity and quality are missing for broilers as well as data about predicted intake.

In temperate regions, forage is commonly stored anaerobically at 50-70% water content in the form of silage. Silage is rich in lactic acid bacteria (LAB) (~10^7 CFU/g fresh matter) and has a low pH (~4). In Nordic countries, it is common to store forages anaerobically at lower 30–50% water content in a fermented product known as haylage. Haylages have generally lower contents of LAB and a higher pH compared to silages. The preservation of the forage in haylage is secured by the low moisture content that prohibits microbial growth. To the best of current knowledge, information about haylage and silage provision as a feed to both organic and conventional broilers remain largely unknown.

Campylobacteriosis is the most commonly reported zoonosis in the EU. According to EFSA, 50–80% of human *Campylobacter jejuni* infections are associated with poultry (EFSA 2010). In Sweden, the mean prevalence of *Campylobacter spp.* is approximately 15% of slaughtered conventional broiler flocks (SVA 2018). However, the prevalence is greater (60%) when chickens have access to outdoor areas, due to increased contact with *Campylobacter spp.* reservoirs, such as wild birds, rodents and flies (Rosenquist et al. 2013).

Fermented feeds with low pH and high numbers of LAB have been shown to reduce the susceptibility to *Campylobacter spp.* colonisation in chickens (Heres et al. 2003). This effect might be explained by different mechanisms. It has been reported that *C. jejuni* survive poorly at a pH below 6 (Axelsson-Olsson et al. 2010). Moreover, some LAB can produce bacteriocins (peptides with antimicrobial properties) that are active against both gram-positive and

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gram-negative bacteria and particularly *Campylobacter spp*. (Neal-McKinney et al. 2012). Furthermore, low pH, provision of *Lactobacillus ssp*. as probiotics and the addition of fibre can cause a change in the gut microbiota composition in favour of a reduced *Campylobacter spp*. abundance. In theory, the use of silage as feed to organic broilers would combine all of these effects. In addition, it has been reported that provision of water inoculated with *L. plantarum* might decrease the level of *C. jejuni* colonisation in the bird's gut (Kobierecka et al. 2017). Supplementing birds' drinking water with LAB in order to inhibit the growth and survival of *C. jejuni*, could be a promising strategy to reduce the load of *C. jejuni* in conventional broiler production.

The aim of the present study was to investigate the effects of daily intake of silage or haylage on broiler production performance and organ development. Furthermore, the effects of LAB intake, either *via* intake of silage/haylage or by supplemented drinking water, on *C. jejuni* loading in faeces at the end of the rearing period were studied. Silage inoculated with the *L. plantarum* strain 256 and haylage without inoculation with *L. plantarum* as a closest control to the silage were used. Effects of the treatments were evaluated in broilers from slow- and fast-growing genotypes in two separate trials after a *C. jejuni* challenge.

Materials and methods

Experimental design

The experiments were carried out at the Swedish Livestock Research Centre of the Swedish University of Agricultural Sciences, located outside Uppsala and were approved by the committee for animal ethics of the Uppsala region (approval number 5.8.18-16 271/2017).

Two trials were conducted in parallel on two different hybrids of broiler chickens. In both experiments, two random chickens from each replicate were individually marked with a neck tag by tagging gun (Jolly Fine, Jolly, Italy) at 11 d of age. These focal birds were later used for the collection of faeces for *C. jejuni* culture and quantification.

Experiment 1 included a total of 160, one-day-old, unsexed, Ross 308 hybrid broiler chickens. The chicks were sourced from hen aged 26 weeks, and had an initial body weight 32.0 ± 0.5 g (mean ± SD). The length of experiment 1 was limited to 42 days, which is considered a normal growing period for fast-growing strains.

Experiment 2 included a total of 160, 1-d-old unsexed Rowan Ranger hybrid broiler chickens. The chicks were sourced from 38 weeks old hens and had an initial body weight 38.1 ± 0.6 g (mean ± SD). The length of experiment 2 was 63 d, in accordance with the age at which slow-growing hybrids are generally slaughtered from organic production systems in Sweden.

Housing and management

In each trial, chickens were randomly distributed in groups of eight in 20 raised pens (1.5 × 0.75 m) with four dietary treatments and five pen replicates for each treatment, arranged in a randomised block design. The trials were conducted during the winter (outside temperature below 0°C) in an insulated house equipped with the facilities for automatic control of light and temperature. Light was provided 24 h/d during the first 2 d and was then reduced by 1 h every day until day eight, giving 18 h of light per day during the remaining period. The temperature was maintained at 33°C for the first 3 d and thereafter gradually reduced according to age until reaching 23°C on d 24, remaining the same for the rest of the period. Body temperature of the birds was checked during the first few days of trials to ensure that they had a stable internal temperature in accordance with their needs. On the chicks' arrival, fresh wood shavings were provided as litter in each pen. Pens were equipped with feeders – metal plates for the starter feed and metal troughs for the grower feed. Water was provided in 3-l bell drinkers.

Forage preservation

Second-cut grass (seeding composition: 70% timothy and 30% meadow fescue) harvested from a field outside Uppsala, Sweden in the last week of September 2017, was used in the production of silage and haylage which differed in dry matter content. Grass for the silage was inoculated with *L. plantarum* 256 during baling, providing an inoculum concentration of 10^8 CFU per gram fresh matter. The strain *L. plantarum* 256 was originally isolated from silage (Johansson et al. 1995) and was chosen after initial *in vitro* evaluation of different LAB. Silage bales with 450 g/kg DM, weighed 655 kg on average and were wrapped with 16 layers of plastic wrap. Haylage bales with 715 g/kg DM weighing 370 kg on average with 10 layers of plastic wrap, were made without inoculation. After 11 weeks of storage, bales were opened and the silages/haylages were chopped to a length of 5–10 cm, followed by a further chopping with an industrial meat grinder to 0.5–1 cm particles. Forage was then vacuum packed using a Genzo ProPack V4 machine (Hylte Jakt and Lantman, Hyltebruk, Sweden), in 1 kg bagged batches. From there on, *i.e*., 2 d prior to the start of the experiments, bags were stored in an uninsulated room (at temperature below 0°C) to maintain a similar feed quality throughout the experiments.

Composition of experimental diets

Birds were provided *ad libitum* daily with fresh feed and water. The base of all the experimental diets were organic-pelleted compound feeds; a crumbled starter from days one to 20 and a 3 mm pellet grower from d 20 and onwards (Table 1). Daily nutritional requirements for the formulation of the pellets was based on the Management Handbook for Ross 308 (Aviagen 2014b) and Rowan Ranger (Aviagen 2017). Daily feed allowances were increased by 25% in all treatment groups to ensure *ad libitum* provision of the feed. Chickens from each breed were divided into four different treatment groups; silage, haylage, LP256 or control. Silage and haylage experimental diets were formulated as total mixed ratios (TMR) containing 85% pellets and 15% of the respective forage (on a DM basis). Hence, on a DM basis 15% of the pellets was replaced by forage. The LP256 and the control groups received the organic-pelleted compound feed (no forage provided). The LP256 group had their drinking water inoculated with *L. plantarum* 256 (10^7 CFU/ml) and the control group received clean, unsupplemented water.

Silage and haylage were thawed overnight in the fridge and mixed with pellets before being provided to the birds as TMR.

Table 1. Diet composition (g/kg as fed) and analysed chemical composition (g/kg DM) of compound feed.

Ingredient (g/kg as fed)	Starter	Grower
Wheat	670	620
Oats	.	120
Soybean expeller	140	70
Fishmeal	70	70
Barley	.	30
Malt sprouts	.	30
Rapeseed cake	40	.
Rapeseed	.	20
Potato protein	30	20
Source of vitamins and minerals permix1,2	30	20
Maize gluten meal	20	.
Total	1000	1000
Analysed chemical composition (g/kg DM)		
Metabolisable energy MJ/kg (calculated)	13.0	13.0
DM	891	891
Ash	56	53
Crude protein	253	230
Crude fibre	38	40
Ether extract	39	40
Water soluble carbohydrates	30	25
Starch	465	482
Lysine	13.6	11.2
Methionine	5.2	4.3
Cysteine	4.3	3.6
Threonine	10.2	8.6

[1]The starter premix provided (per kg diet): retinyl acetate: 13,500 IU; cholecalciferol: 4,100 IU; dl-α-tocopherol acetate: 75 mg; betaine: 980 mg; Fe: 27 mg; Cu: 8 mg; Mn: 95 mg; Zn: 108 mg; I: 2.7 mg; Se: 0.47.
[2]The grower premix provided (per kg diet): retinyl acetate: 10,000 IU; cholecalciferol: 3,000 IU; dl-α-tocopherol acetate: 50 mg; betaine: 980 mg; Fe: 20 mg; Cu: 6 mg; Mn: 70 mg; Zn: 80 mg; I: 2.0 mg; Se: 0.35.

Feed residues were collected and measured daily from each group. In each experiment, water intake was estimated daily from two pens per treatment, calculated as the difference between the amount of water provided and the remainder.

Experimental feed analyses and energy calculations

Pellets and silage/haylage were analysed for DM (Table 1) by drying at 103°C for 16 h and then ashed by ignition at 600°C for 3 h (Jennische and Larsson 1990). The content of crude protein (N × 6.25) was determined by the Kjeldahl method (NMKL 2003), and the crude fibre was analysed according to the method of Jennische and Larsson (1990). The fat (as ether extract; EE) in feed was determined according to Official Journal of European Communities (1994). Starch, including maltodextrins, was analysed by an enzymatic method described by Larsson and Bengtsson (1983), whereby free glucose was determined separately and subtracted from the starch value. Water-soluble carbohydrates (WSC), including glucose, fructose, sucrose and fructans, were determined using enzyme-based acid hydrolysis as reported by Larsson and Bengtsson (1983). In addition, amino acid composition of the starter and grower feed was analysed according to ISO (2005) methods.

The metabolisable energy (ME) of the compound feed was calculated using the formula:

ME (MJ/kg) = 0.1551 × % crude protein + 0.3431 × % crude fat + 0.1669 × % starch + 0.1301 × % total sugar (Commission Regulation (EC) 152/2009)

The ME of forage was calculated according to a method presented by WPSA (Janssen 1989) based on digestible nutrients.

To enumerate silage/haylage LAB, 50 g sample was macerated in 450 ml Ringer solution (Merck KGaA, Darmstadt, Germany) for 2 min in a laboratory stomacher after which, serial dilution was made from the microbial suspension. Cultivation was done using the pour-plate method on Man, Rogosa and Sharpe (MRS) agar (Merck KGaA, Darmstadt, Germany) plates and colonies were counted after 72 h anaerobic incubation at 30°C. The enumeration was carried out 3 d prior to the experiments and, thereafter, once per month. After grinding the silage/haylage in a meat grinder, silage/haylage juice was extracted and pH was measured using a pH metre (Metrohm 654; Metrohm AG, Herisau, Switzerland).

Campylobacter jejuni colonisation

In order to study the effects of the dietary treatments on *C. jejuni* colonisation in the gut, all chickens were orally infected at 22 d of age in Experiment 1 and 29 d of age in Experiment 2. At the day of infection, 0.5 l of water containing 10^6 CFU/ml of the *C. jejuni* strain #65 (ST-104, in ST-21 CC); isolated from a broiler chicken in the UK in 2006, was supplied in the bell drinker of each pen. The inoculated water was provided for 3 h and viability of *C. jejuni* in the water was determined by culturing at the start and end of the 3-h challenge.

Faecal sampling and plating for *C. jejuni* quantification

For faecal sampling, two focal birds from each pen were placed individually in clean boxes for a maximum of 20 min. Sterile plastic loops were used to collect faecal matter from the bottom of the box. Faecal samples were taken from all birds 1 d before infection challenge, to ensure that the birds were culture negative for *Campylobacter* before inoculation. In experiment 1, samples were taken from identified birds at 19-d post-infection (d.p.i), i.e. at 41 d of age, the before the end of the trial. In Experiment 2, samples were taken at 33 d.p.i, i.e. at 62 d of age, the day before the end of the trial.

Approximately 100 mg of fresh faecal matter was collected per bird and re-suspended in 1 ml Luria-Bertani (LB) medium complemented with 20% glycerol. Samples were vortexed and centrifuged (100 × *g* for 15 s) in order to create a pellet of the faecal matter. Thereafter, 100 µl was withdrawn and serially diluted 10-fold, plated on modified charcoal cefoperazone deoxycholate agar plates (mCCDA) and incubated for 26 h at 42°C under microaerobic conditions (Campygen, Thermo Fisher, USA). After incubation, colonies were counted on the plate corresponding to the dilution that gave approximately 100 CFU per plate.

Production parameters, organ weights and foot-pad scoring

Calculations regarding feed intake (FI) of pellets and forage were done on a DM basis. TMR residues were separated using a JEL 200-II sieve with a 2 mm mesh (J. Engelsmann AG, Ludwigshafen, Germany). Residual pellets and forage were subtracted from provided amount and divided by the number of chickens in pens. Feed conversion ratio (FCR) calculations were done on a DM basis and were corrected for mortality. Dead birds were recorded, weighed and removed from pens

daily. At 14 d of age, all chickens were weighed and, in each experiment, chickens with a live weight more than two times standard deviation (SD) lower than the mean were culled due to poor weight gain.

At 21 and 42 d of age in Experiment 1, one random chicken from each pen was selected and killed by an intravenous injection of sodium pentobarbital through the wing vein. The body weight (BW) and weight of internal organs were noted. Weight of gizzard with contents (full) and without (empty), intestines with pancreas, empty small and large intestine, heart, liver and proventriculus were recorded. The length of the small and large intestine, colon and caeca was measured. The same procedure was performed in experiment 2 at 28, 42 and 63 d of age. Moreover, the inner surfaces of the empty gizzards were scored on a 4-point scale from 1 (poor condition) to 4 (good condition) at 42 and 63 d of age for each experiment, respectively. The foot-pads of the selected birds (both feet) were examined for lesions at the end of the respective experiments, according to Ekstrand et al. (1998).

Statistical analyses

Statistical analyses of production performance and organ data were performed with the Proc Mixed procedure in SAS (SAS Institute 2013) to determine treatment effects by one-way analysis of variance (ANOVA). The model included treatment as a fixed factor and pen served as the experimental unit for performance data. Organ measurements were determined repeatedly with age as an additional fixed factor, using a repeated statement with unstructured covariance matrix. Gizzard surface scores were analysed by the Glimmix procedure in SAS, with treatment as a fixed factor and pen as a random factor, where a binary logistic model was used to evaluate if gizzard surface was affected by the treatment. Prior to the analyses, scoring values of 1, 2 or 3 were converted to binary value 1 and scoring value 4 (good condition) to the binary value 0. The proportion of dead birds was analysed with the Glimmix procedure with pen and treatment as a fixed factors, where the binary logistic model was used to evaluate if mortality appeared (1) or not (0). Plating results were evaluated by one-way ANOVA test, and statistical analysis was performed using GraphPad Prism 6. The probability value, which denotes statistical significance was $P \leq 0.05$. Results were presented as least square means (LSMeans) with a pooled standard error of means (SEM), unless otherwise stated.

Results

Forage parameters

Silage, on a DM basis, contained 238 g/kg crude fibre, 102 g/kg WSC, DM content was 450 g/kg and calculated ME 3.3 MJ/kg. Haylage (DM basis) contained 247 g/kg crude fibre, 108 g/kg WSC, DM content was 715 g/kg and calculated ME (MJ/kg DM) 3.2 MJ/kg (Table 2).

Monthly enumeration of LAB showed that silage contained 8.0, 7.4 and 7.2 log (cfu/g) of LAB, respectively, while haylage had 5.0, 3.8 and 3.0 log cfu/g. Hence, silage displayed ≥3 x 10-log (cfu/g) higher LAB concentrations than haylage and a gradual decrease in LAB concentrations was observed in both forages. The pH measurement prior to the experiment was pH 4.4 for silage and pH 6.2 for haylage.

Table 2. Analysed chemical composition of silage and haylage (g/kg DM).

Nutrient	Silage	Haylage
Metabolisable energy MJ/kg DM (calculated)	3.3	3.2
DM	4501	715
Ash	75	86
Crude protein	99	102
Crude fibre	238	247
Ether extract	31	21
Water soluble carbohydrates	102	108
Free (glucose + fructose)	68	85
Starch	13	12

[1]In-house corrected dry matter to compensate for lost volatiles during drying (Mogodiniyai Kasmaei, 2014).

Production performance

In Ross 308 birds (experiment 1), dietary treatment affected growth and feed intake (Table 3). At seven and 14 d of age, accumulated BW was higher in the LP256 groups, intermediate in the control and silage, and inferior in the haylage groups. By the end of the trial, at 42 d of age, there was no difference in BW between control and LP256 groups, but BW was significantly lower in the silage groups and even lower in the haylage groups. Inclusion of haylage in the TMR had an adverse effect on feed intake throughout the trial, when compared to the other dietary treatment groups. Besides one exception at 28 d of age, there were no differences in accumulated FI between the control, silage or LP256 groups. Differences in FCR between groups were observed at most of the time points, but none of these remained at 42 d of age.

In the Rowan Ranger birds (Experiment 2), there were no significant differences between diets either in BW or FI (Table 3) but there were some tendencies ($P < 0.10$). Birds provided with L. plantarum 256 in the water had a tendency for the highest BW at 7 d of age in comparison with other groups (Table 3). The same pattern was observed for the feed intake, where at 14 and 28 d of age, there was a tendency for higher FI in LP256 groups. There were differences in FCR between groups in the latter part of the experimental period. At the end of the experiment at 63 d of age, FCR was lower in control compared to the haylage and LP256 groups, but not different from the silage group.

In experiment 1, the lowest water intake (Table 3) was observed in the haylage group in comparison with other groups, suggesting that the water consumption corresponded to feed intake. No significant differences in water intake were observed in experiment 2.

Mortality and culling

No significant differences in the proportion of dead birds between the groups were observed in either Ross 308 (experiment 1) or Rowan Ranger (experiment 2). The actual mortality was 13 and five dead birds in experiment 1 and experiment 2, respectively. At 14 d of age, the mean weight of Ross 308 birds was 225 g (SD 63 g), and five birds were culled according to the culling criteria described above. Rowan Ranger mean weight was 240 g (SD 55 g), three chickens were culled.

Intake of pellets and forage

The average daily intake of haylage on a DM basis was 58 and 50 g per bird in experiment 1 and experiment 2, respectively, corresponding to 11% and 8% of the feed intake. The intake of silage on a DM basis represented 14% of feed provided in

Table 3. Weekly accumulated BW, accumulated feed intake (FI) on DM basis and calculated FCR on DM basis. Water intake per bird (mL) and cumulative mortality. Least square means ± pooled SEM (unless other is stated).

	Experiment 1 (Ross 308)						Experiment 2 (Rowan Ranger)					
Item	C[1] n = 5	H[2] n = 5	S[3] n = 5	LP256[4] n = 5	Pooled SEM	P-value	C[1] n = 5	H[2] n = 5	S[3] n = 5	LP256[4] n = 5	Pooled SEM	P-value
BW (g)												
d 7	108^{b}	86^{c}	106^{b}	119^{a}	2.72	<.0001	102	100	105	115	3.90	0.060
d 14	264^{b}	200^{c}	255^{b}	307^{a}	13.30	0.0003	234	231	248	282	17.59	0.188
d 21	627^{a,b}	423^{c}	574^{b}	692^{a}	31.17	<.0001	468	452	482	549	32.65	0.213
d 28	1141^{a}	827^{c}	987^{b}	1203^{a}	37.48	<.0001	762	729	784	851	38.04	0.180
d 35	1793^{a,b}	1352^{c}	1633^{b}	1887^{a}	60.01	<.0001	1256	1146	1163	1300	43.50	0.071
d 42	2509^{a}	1960^{c}	2256^{b}	2588^{a}	82.42	0.0003	1739	1578	1648	1713	56.69	0.225
d 49	.	.	.	.	.	.	2299	2027	2159	2166	71.22	0.106
d 56	.	.	.	.	.	.	2879	2543	2697	2705	127.19	0.083
d 63	.	.	.	.	.	.	3291	3005	3159	3102	92.05	0.214
FI (g)												
d 7	102^{a}	93^{b}	115^{a}	112^{a}	3.45	0.0017	104	94	106	105	4.56	0.187
d 14	453^{a,b}	367^{c}	447^{b}	484^{a}	14.42	<.0001	335	305	334	365	13.75	0.054
d 21	918^{a}	711^{b}	906^{a}	997^{a}	43.22	0.0002	645	641	684	772	42.41	0.151
d 28	1635^{a,b}	1258^{c}	1491^{b}	1760^{a}	79.84	0.0007	1177	1110	1176	1359	62.64	0.069
d 35	2499^{a}	2184^{b}	2526^{a}	2640^{a}	117.12	0.0225	1845	1772	1878	2031	80.11	0.181
d 42	3735^{a}	3115^{b}	3553^{a}	3930^{a}	153.41	0.0025	2763	2556	2679	2944	105.01	0.108
d 49	.	.	.	.	.	.	3713	3530	3674	3752	154.40	0.761
d 56	.	.	.	.	.	.	4835	4642	4834	4797	201.34	0.889
d 63	.	.	.	.	.	.	5914	5731	5950	5809	228.28	0.901
FCR												
d 7	1.66^{b,c}	2.07^{a}	1.84^{a,b}	1.54^{c}	0.08	0.0027	1.96	1.70	1.80	1.53	0.10	0.052
d 14	2.20^{a,b}	2.43^{a}	2.23^{a}	1.95^{b}	0.09	0.0148	1.98	1.86	1.87	1.72	0.11	0.472
d 21	1.71^{b}	1.90^{a}	1.85^{a}	1.67^{b}	0.04	0.0004	1.74	1.78	1.77	1.73	0.04	0.748
d 28	1.82	1.96	1.85	1.82	0.06	0.1550	1.82	1.84	1.83	1.88	0.02	0.397
d 35	1.69^{b}	1.90^{a}	1.90^{a}	1.67^{b}	0.04	<.0001	1.87	1.93	2.08	1.97	0.05	0.080
d 42	1.82	1.89	1.89	1.85	0.05	0.3366	1.80^{B}	1.91^{A}	1.90^{A}	1.96^{A}	0.03	0.005
d 49							1.88^{B}	2.03^{A}	1.98^{A,B}	2.01^{A}	0.04	0.039
d 56							1.98^{B}	2.16^{A}	2.11^{A}	2.09^{A,B}	0.04	0.022
d 63							2.07^{B}	2.21^{A}	2.18^{A,B}	2.22^{A}	0.04	0.045
Water intake	210^{a}	165^{b}	205^{a}	226^{a}	8.14	0.042	196	200	201	209	22.54	0.981
Mortality (%)[5]	4	8	8	2	0.19	0.24	4	2	4	0	0.15	0.899

[1]C = control feed; [2]H = diet based on 85% of pellets and 15% of haylage; [3]S = diet based on 85% of pellets and 15% of silage; [4]LP256 = control feed and water inoculated with 10^{7} c.f.u/ml of viable *L. plantarum* 256; [5]Mortality (dead and culled birds) results are presented as means.
[a-c]Least square means within the same row (Experiment 1) with different superscripts were significantly different ($P < 0.05$).
[A-B]Least square means within the same row (Experiment 2) with different superscripts were significantly different ($P < 0.05$).

experiment 1 and 10% in experiment 2, with 86 and 67 g of average daily intake per bird in experiment 1 and experiment 2, respectively.

In Ross 308 birds (experiment 1) the intake of pellets was, for most weeks, higher in LP256 and control groups compared to the haylage and silage groups (Table 4).

In Rowan Ranger birds, differences in weekly intake of pellets were only observed on d 14, d 28 and d 42, with intake of pellets being higher in the control and LP256 groups than in the haylage and silage groups. The intake of silage was higher than haylage in experiment 1 at seven, 14, 21 and 28 d of age. The same was observed in experiment 2 at seven and 14 d of age (Table 4).

Organ measurements

In Ross 308 birds (experiment 1), decreasing relative organ weight (ROW) with age was observed for all evaluated organs (Table 5). ROW of gizzard full was higher in haylage and silage groups in comparison to control and LP256 group. The ROW of empty gizzards was higher in haylage-fed birds than in birds in the control or LP256 groups, whereas silage groups were intermediate. The ROW of intestines with pancreas and the empty small intestine were higher in birds fed haylage in comparison to the LP256 group, and intermediate in the control and silage groups. Tendencies ($P < 0.10$) were shown for ROW of the empty large intestine and length of small intestine and caeca, respectively, due to higher relative weight or length of the respective organs related to the haylage and silage treatments.

Relative organ length (ROL) of the large intestine and the colon were higher in birds fed haylage in comparison to birds with LP256, and intermediate in the birds from the control and silage groups.

In Rowan Ranger birds (experiment 2), ROW decreased with age in agreement with the results from experiment 1. The only significant difference in weight and length of organs between groups were in empty gizzards, where ROW was higher in birds fed silage in comparison to the control and LP256 groups, but not different from haylage (Table 5). Birds fed silage had a tendency for the higher ROW of full gizzard in comparison with birds from the other groups.

Gizzard surface and foot-pad scores

In both experiments, the gizzard surfaces of chickens were not significantly affected ($P > 0.05$) by dietary treatments (data not shown here and henceforth in this article). In experiment 1, at 42 d of age, 35% of the scored chickens had gizzards with a condition considered 'good' (score 4) whereas 65% of chickens had gizzards in an inferior condition regarding their inner surface (scores 1 – ulcers and surface changes seen, 2 – surface changes or 3 – surface irritation). In experiment 2, at 42 d of age, 55% of the chickens had a gizzard score of 4, implying good condition, and 45% had an inferior gizzard surface conditions.

Table 4. Actual weekly intake of pellets and roughage (silage or haylage) on DM basis per bird. Least square means ± pooled SEM.

	Experiment 1 (Ross 308)						Experiment 2 (Rowan Ranger)					
Item	C[1] n = 5	H[2] n = 5	S[3] n = 5	LP256[4] n = 5	Pooled SEM	P-value	C[1] n = 5	H[2] n = 5	S[3] n = 5	LP256[4] n = 5	Pooled SEM	P-value
Pellets (g)												
d 7	102[b]	93[c]	106[a,b]	112[a]	2.57	0.001	104	93	96	105	4.37	0.181
d 14	349[a]	258[c]	295[b]	370[a]	12.40	<.0001	230[B]	195[C]	196[C]	260[A]	9.01	0.003
d 21	464[a,b]	349[c]	420[b]	512[a]	30.92	0.002	310	316	311	406	27.46	0.068
d 28	732[a]	488[b]	506[b]	778[a]	37.24	<.0001	532[A]	414[B]	433[B]	587[A]	21.16	<.0001
d 35	815	770	868	836	43.40	0.404	682	616	643	685	33.04	0.097
d 42	1222[a]	803[b]	879[b]	1276[a]	47.43	<.0001	913[A]	701[B]	724[B]	918[A]	28.40	<.0001
d 49	.	.	.	.	.	.	976	928	920	832	89.45	0.472
d 56	.	.	.	.	.	.	1122	1046	1049	1045	61.39	0.772
d 63	.	.	.	.	.	.	1079	1011	1007	1012	28.41	0.262
Roughage (g)												
d 7	.	0[b]	9[a]	.	0.23	<.0001	.	1[B]	10[A]	.	0.64	<.0001
d 14	.	11[b]	39[a]	.	2.28	<.0001	.	17[B]	31[A]	.	3.72	0.035
d 21	.	0[b]	38[a]	.	3.31	<.0001	.	22	38	.	7.09	0.161
d 28	.	76[b]	102[a]	.	6.55	0.003	.	54	59	.	8.08	0.676
d 35	.	128	161	.	18.99	0.099	.	59	71	.	11.78	0.514
d 42	.	133	164	.	19.55	0.127	.	83	77	.	15.19	0.760
d 49	.	.	.	.	.	.	.	70	97	.	16.96	0.287
d 56	.	.	.	.	.	.	.	65	112	.	16.26	0.080
d 63	.	.	.	.	.	.	.	78	109	.	21.57	0.335

[1]C = control feed; [2]H = diet based on 85% of pellets and 15% of haylage; [3]S = diet based on 85% of pellets and 15% of silage; [4]LP256 = control feed and water inoculated with 107 c.f.u/ml of viable *L. plantarum* 256.
[a-c] Least square means within the same row (Experiment 1) with different superscripts were significantly different (P < 0.05).
[A-C] Least square means within the same row (Experiment 2) with different superscripts were significantly different (P < 0.05).

Table 5. Relative organ weights (ROW) presented as g/kg BW and relative organ length (ROL) in correlation to body weight presented as cm/kg BW. Least square means ± pooled SEM.

	Age (days)			Treatment				Pooled SEM		P-value		
Experiment 1	21	42		C[1] n = 5	H[2] n = 5	S[3] n = 5	LP256[4] n = 5	A[5]	T[6]	A[5]	T[6]	A*T[7]
Gizzard full	3.93	2.25		2.42[b]	3.88[a]	3.45[a]	2.63[b]	0.17	0.22	<.0001	<.0001	0.144
Gizzard empty	2.76	1.41		1.81[b]	2.52[a]	2.26[a,b]	1.75[b]	0.11	0.16	<.0001	0.004	0.787
Intestines + pancreas	9.15	5.79		6.93[b,c]	8.39[a]	8.02[a,b]	6.55[c]	0.27	0.37	<.0001	0.002	0.490
Heart	0.77	0.60		0.72	0.67	0.68	0.66	0.03	0.03	<.0001	0.495	0.341
Liver	4.01	2.14		3.25	3.09	3.19	2.76	0.21	0.25	<.0001	0.318	0.390
Proventriculus	0.69	0.42		0.58	0.59	0.54	0.50	0.02	0.03	<.0001	0.161	0.484
Small intestine empty	4.36	2.49		3.35[a,b]	3.72[a]	3.49[a,b]	3.13[b]	0.08	0.12	<.0001	0.013	0.439
Large intestine empty	0.71	0.51		0.61	0.65	0.65	0.52	0.03	0.04	<.0001	0.059	0.867
ROL (cm/kg BW)												
Small intestine	21.7	7.58		14.0	16.9	15.0	12.6	0.61	0.86	<.0001	0.057	0.402
Large intestine	5.08	2.09		3.45[a,b]	4.05[a]	3.77[a,b]	3.07[b]	0.13	0.19	<.0001	0.005	0.153
Colon	1.30	0.45		0.84[a,b]	1.03[a]	0.87[a,b]	0.75[b]	0.05	0.06	<.0001	0.008	0.454
Caeca	3.83	1.67		2.61	3.01	2.93	2.46	0.14	0.18	<.0001	0.064	0.165
Experiment 2	28	42	63	C[1] n = 5	H[2] n = 5	S[3] n = 5	LP256[4] n = 5	A[5]	T[6]	A[5]	T[6]	A*T[7]
ROW (g/kg BW)												
Gizzard full	5.02[A]	3.76[B]	2.47[C]	3.31	3.94	5.07	3.68	0.53	3.75	<.0001	0.067	0.586
Gizzard empty	3.03[A]	2.30[B]	1.51[C]	2.07[c]	2.38a-c	2.55[a]	2.13[b,c]	2.28	2.28	<.0001	0.003	0.931
Intestines + pancreas	8.56[A]	7.91[A]	5.74[B]	8.43	7.70	7.17	7.31	0.92	0.93	<.0001	0.746	0.597
Heart	0.75[A]	0.71[A]	0.54[B]	0.68	0.66	0.65	0.68	0.06	0.06	<.0001	0.816	0.809
Liver	4.09[A]	3.87[A]	2.90[B]	3.71	3.40	3.54	3.82	0.32	0.34	<.0001	0.142	0.430
Proventriculus	0.75[A]	0.63[B]	0.50[C]	0.61	0.61	0.65	0.64	0.09	0.09	<.0001	0.761	0.716
Small intestine empty	3.58[A]	2.89[B]	2.03[C]	2.81	2.94	2.82	2.76	0.22	0.22	<.0001	0.671	0.580
Large intestine empty	0.84[A]	0.80[A]	0.64[B]	0.76	0.78	0.74	0.75	0.07	0.06	<.0001	0.715	0.920
ROL (cm/kg BW)												
Small intestine	21.6[A]	13.9[B]	9.6[C]	15.3	15.0	15.2	14.7	1.30	1.32	<.0001	0.818	0.883
Large intestine	5.07[A]	3.32[B]	2.24[C]	3.54	3.60	3.57	3.47	0.35	0.36	<.0001	0.913	0.579
Colon	1.31[A]	0.83[B]	0.61[C]	0.89	0.90	0.93	0.94	0.12	0.12	<.0001	0.904	0.905
Caeca	3.78[A]	2.53[B]	1.64[C]	2.66	2.73	2.68	2.55	0.26	0.26	<.0001	0.601	0.455

[1]C = control feed; [2]H = diet based on 85% of pellets and 15% of haylage; [3]S = diet based on 85% of pellets and 15% of silage; [4]LP256 = control feed and water inoculated with 10^7 c.f.u/ml of viable *L. plantarum 256*; [5]A = age effect; [6]T = treatment effect; [7]A*T = age*treatment effect.
[a-c] Least square means within the same row (Experiment 1) with different superscripts were significantly different (*P* < 0.05).
[A-C] Least square means within the same row (Experiment 2) with different superscripts were significantly different (*P* < 0.05).

However, at 63 d of age, all Rowan Ranger birds had gizzards in good condition. The foot-pad examination for lesions at the end of each experiment did not show any effect of treatments, since all birds were graded as class 0, denoting no lesions.

Culture determined colonisation patterns of *C. jejuni*

The evaluation of C. *jejuni* in faecal samples prior to the *C. jejuni* challenge showed that both hybrids were negative, i.e. not colonised. Subsequent colonisation with 10^6 cfu of *C. jejuni* per ml in drinking water was successful in both

experiments. Quantification of *C. jejuni* in faecal samples by the end of each experiment showed no significant differences in *C. jejuni* colonisation levels between the treatments in either of the experiments.

Discussion

In the present trials, one of the aims was to study how a daily intake of silage and haylage affected the performance and organ development of both slow- and fast-growing broiler chickens. Currently, there appears to be a shortage of studies focusing on the provision of forages based on grass to broiler chickens, even though the provision of forage is a requirement in organic poultry production (Commission Regulation (EC) 889/2008). Negative effects of haylage on BW and FI in comparison with birds fed only feed pellets were observed, particularly in Ross 308 birds (experiment 1). Haylage-fed Ross 308 birds had significantly lower BW than birds in the control group at 42 d post hatch, weight difference being 549 g, representing 22% lower BW. Birds fed only pellets (control and LP256 groups) weighed around 2.5 kg at 42 d of age, which was 300 g less than the predicted growth according to the Performance Objectives for Ross 308 broilers (Aviagen 2014a). However, the predicted performance stated in the Performance Objectives was based on Ross 308 birds in a commercial setting. The lower growth performance in the present study was probably due to the provision of organic feed, which has a different nutrient composition than conventional feed. Moreover, the low weight of Ross 308 1-d-old chickens due to the young age of their dams may have been an influencing factor. Lower FI and BW were observed in the haylage-fed Ross 308 birds in comparison to those fed silage, indicating that haylage reduced voluntary feed consumption and, in turn, growth performance. The results showed higher consumption of silage than haylage in most weeks, and one explanation could be the drier texture of haylage particles due to higher DM content, which likely decreased the bird's interest in the feed. Thus, birds probably learnt to visually avoid substances that caused unpleasant post-ingestion effects (Gillette et al. 1983). It is noteworthy that haylage did not have a significant adverse effect either on FI or on BW of the Rowan Ranger birds (experiment 2).

Unlike a previous experiment on feeding poultry with maize silage as supplemental foraging material (Steenfeldt et al. 2007), the present study provided grass silage and haylage as a TMR with pellets, which likely enabled higher forage intake as compared to feeding forage separately. This was to avoid the obvious risk that the pelleted concentrate would have been preferred if fed separately. The short length of chopped forage in TMR better enabled forage feeding to the day-old chickens. Longer fibre length might have induced problems, such as crop and gizzard impaction (Christensen 1998). The provision of foraging material in a TMR in the present study decreased the intake of pellets in the haylage group in the first half of experiment 1, which may be explained by the fast-growing broilers sensitivity to dietary quality and structure of the feed (Tufarelli et al. 2018). Ranjitkar and Engberg (2016) reported that Ross 308 broilers fed a pelleted diet with 15% inclusion of crimped kernel maize silage (CKMS) on a DM basis (fed as TMR) had comparable FI with the control group. This is in agreement with the current findings in Ross 308 birds (experiment 1), where silage-fed birds had similar FI when compared to the control. Higher intakes of silage in the study by Ranjitkar and Engberg (up to 30% of supplemented silage) could be attributed to the different nutritional composition of maize silage when compared to grass silage, especially regarding the higher content of ME in maize silage.

Intake of water plays an important role in commercial broiler management, since it influences quality of the carcase as well as conditions of the litter (Jiménez-Moreno et al. 2016). The lowest water intake was seen in the Ross 308 birds (experiment 1) was observed in the haylage groups, suggesting that water consumption corresponded to the lowest FI observed in these groups.

Probiotics are live microorganisms that contribute to the health and balance of the host digestive system (Fuller 1989). Karimi Torshizi et al. (2010) reported that the probiotic administration method affects its efficiency, where provision by drinking water was found to be the most effective. They administered a probiotic supplement consisting of nine different microorganisms (Protexin, UK) in water for Ross 308 chickens and reported increased BW, higher FI in the starter period and lower FCR in groups with the probiotic compared to the unsupplemented, control group. This is in agreement with the current findings whereby increased BW was seen in Ross 308 birds given drinking water inoculated with *L. plantarum* 256 (experiment 1) in the starter period. The discrepancies in FI and FCR results may be caused by microorganisms other than *L. plantarum* in the probiotic supplement, which was a probable environmental management effect.

It is well known that the physical structure of the feed effects the physiology and morphology of the gastrointestinal tract (GIT) in birds (Engberg et al. 2002). As expected, the increased intake of fibre in chickens fed a TMR with forage inclusion seen in these experiments had clear effects on the relative weight of some GIT organs in both fast- and slow-growing genotypes. The weight of empty gizzards was higher in birds fed haylage and silage than in the control group of both in Ross 308 and Rowan Ranger birds. In Ross 308 birds, both forages increased the weight of full gizzards when compared to the birds fed only pellets. These results were consistent with data from González-Alvarado et al. (2008), who observed increased relative weight of the gizzards as well as the digesta content of the gizzards when fibre was included in the diet. Nonetheless, they concluded that both the source and particle size of fibre were important, since 3% oat hull inclusion (467 µm) resulted in a 32% increase of gizzard size, while the same inclusion level of soy hulls (582 µm) did not affect the size of the gizzard. The reaction of birds to fibre inclusion could be explained by the findings of Mateos et al. (2012), stating that the response to fibre inclusion is dependent on its amount and source, as well as on the physiological state of the broilers.

Gizzard erosion and ulceration (GEU) syndrome is a widely spread, subclinical condition in commercial poultry flocks. GEU syndrome can be induced by feed structure, nutritional deficiencies or microbial colonisation. Yet, knowledge about the definitive cause of the syndrome is lacking (Gjevre et al. 2013). In both current experiments, GEU was observed, with a higher incidence in Ross 308 birds (experiment 1). The reason for different GEU severity among the breeds is not known. Interestingly, no dietary treatment effect was seen, even though the drier texture of haylage would be expected to cause this. At the end of experiment

2, no birds showed affected gizzards, which likely indicated gizzard irritation in the first part of the experiment and possible ability of birds to reverse this later on.

Intensive selection in fast-growing broilers has resulted in increased muscularity and growth with additional adverse effects, including delayed development of the internal organs, which may be the potential cause of several metabolic disorders such as ascites or sudden death syndrome (Dou et al. 2017). The birds in the current studies did not show any signs of these metabolic disorders, although the differences in internal organ size were detected. In contrast to what was observed for the Ross 308 birds, Rowan Ranger internal organs represented a higher percentage of the body weight at 42 d of age. The probable explanation is the lower degree of selection for high growth in organic hybrids.

Footpad dermatitis is a condition causing necrotic lesions on growing broiler's footpads and it is considered an animal welfare issue. No issue regarding foot-pad score were observed in any treatment, indicating appropriate environmental conditions in both experiments. Additionally, birds were kept in small groups with low stocking density and good litter conditions, which is correlated to a lower risk of lesions. Shepherd and Fairchild (2010) defined litter moisture and stocking density as significant predisposing factors in the development of footpad lesions. Moreover, several studies showed that litter material and management are critical factors in maintaining optimum footpad and bird health.

It is well documented that fast-growing broiler hybrids have a higher feed intake than slow-growing ones. Therefore, the numerically higher intake of forage observed for Ross 308 compared to Rowan Ranger birds was expected. In theory, it is reasonable that a possible inhibitory effect of silage on *C. jejuni* colonisation would be related to the level of daily consumption of silage. For that reason, it was of interest to test the effects of silage also on a fast-growing hybrid. However, according to the current studies, grass-based silage inoculated with *L. plantarum* 256 was not an efficient means for reducing *C. jejuni* colonisation in the broilers' gut, at least not at the end of the rearing period.

The current results were in accordance with findings from Ranjitkar and Engberg (2016), who concluded that there was no significant influence on the intestinal colonisation by *C. jejuni* in Ross 308 broilers when crimped kernel maize silage was included in the pelleted maize-based diet. A possible explanation could be that insufficient amounts of silage was consumed in order to manifest a *Campylobacter* reducing effect by lowering the pH in the GIT or to induce changes in the gut microbiota composition. Moreover, the *Lactobacillus* strain *L. plantarum* 256 used in the present study does not produce bacteriocins. However, optimisation such as supplying different *Lactobacillus* strains that thrive in silage while having a stronger inhibitory effect against *C. jejuni* in the bird's intestines, might be a promising approach. The other alternative could be the inoculation of grains with a *Lactobacillus* strain producing bacteriocins, where higher feed intake of birds (up to 60%) can be expected.

In conclusion, the inclusion of 15% of silage or haylage in an organic-pelleted diet (fed as TMR) is possible in slow-growing Rowan Ranger chickens without interfering with performance. When 15% of haylage was included in the diet of the fast-growing hybrid Ross 308, adverse effects on feed intake and body weight were observed during the whole experimental period, while the negative effect of silage inclusion on BW was observed only at weeks four and six of age. Interestingly, water inoculated with *L. plantarum* 256 increased body weight of Ross 308 chickens in the starter period. However, intake of *L. plantarum* 256 *via* silage or inoculated water was not an effective intervention against *C. jejuni* colonisation at the end of the rearing period either in Ross 308 or in Rowan Ranger hybrids. However, further experiments may optimise this approach for better effects.

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Intestinal colonization with *Campylobacter jejuni* affects broiler gut microbiota composition but is not inhibited by daily intake of *Lactiplantibacillus plantarum*

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Introduction: Lactobacilli may prevent broilers from colonization with *Campylobacter* spp. and other gram-negative zoonotic bacteria through lactic acid production and modulation of the intestinal microbiota. This study evaluated the effects of daily intake of *Lactiplantibacillus plantarum* 256 (LP256) on *Campylobacter jejuni (C. jejuni)* loads in ceca and feces of *C. jejuni* challenged broilers, together with the changes in the gut microbiota.

Methods: Two experiments were conducted using the broilers Ross 308 (R-308; Experiment 1) for 42days and Rowan Ranger broilers (RR; Experiment 2) for 63days. The *LP256* strain was administered either via silage inoculated with *LP256* or direct supplementation in the drinking water. Concurrently, haylage as a forage similar to silage but without any inoculum was tested. *C. jejuni* loads in fecal matter and cecal content were determined by plate counts and qPCR, respectively. The cecal microbiota, in response to treatments and the challenge, were assessed by 16S rRNA sequencing.

Results and Discussion: Culturing results displayed a significant reduction in *C. jejuni* colonization (2.01 log) in the silage treatment in comparison to the control at 1 dpi (day post-infection) in Experiment 1. However, no treatment effect on *C. jejuni* was observed at the end of the experiment. In Experiment 2, no treatment effects on *C. jejuni* colonization were found to be statistically significant. Colonization load comparison at the peak of infection (3 dpi) to that at the end of the trial (32 dpi) revealed a significant reduction in *C. jejuni* in all groups, regardless of treatment. Colonization dynamics of *C. jejuni* in the cecal samples analyzed by qPCR showed no difference between any of the treatments in Experiment 1 or 2. In both experiments, no treatment effects on the cecal microbiota were observed. However, proportional changes in the bacterial composition were observed after the *C. jejuni* challenge, suggesting that colonization affected the gut microbiota. Overall, the daily intake of *LP256* was not effective in reducing *C. jejuni* colonization in either broiler type at the end of the rearing period and did not cause any significant changes in the birds' cecal microbiota composition.

KEYWORDS

Lactobacillus plantarum, *Campylobacter jejuni*, broiler, gut microbiota, qPCR, sequencing

1. Introduction

The ceca are believed to have an important role in the gut health and performance of broiler birds. However, its role in the maintenance of gut health and modulation of the gut microbiota is still not fully understood. As the most densely colonized microbial habitat in broilers, its microbial density is estimated to be 10^{11}–10^{12} bacterial cells per gram (Rinttilä and Apajalahti, 2013). The description and understanding of intestinal microbial communities and their interactions, are essential for the development of feed additives and dietary changes to improve broiler health, performance, and welfare (Sugiharto, 2016). A wide variety of feed supplements, such as prebiotics, probiotics, and organic acids, focus on the stabilization of the gut microbiota to secure intestinal health (Yang et al., 2009).

Probiotics are natural microbes that benefit their host fundamentally through their action in the gastrointestinal tract (Abd El-Hack et al., 2020). Single-strain probiotic species including, among others, species of *Bifidobacterium*, *Bacillus*, *Enterococcus*, *Streptococcus*, and *Lactobacillus* have previously shown positive effects on broiler performance, modulation of the gut microbiome as well as inhibition of pathogens through different principles, i.e., competitive exclusion, production of organic acids, or production of antimicrobial compounds (Neal-McKinney et al., 2012; Prabhurajeshwar and Chandrakanth, 2019; Krysiak et al., 2021). Furthermore, it has been shown that probiotics help to maintain microbial homeostasis thus avoiding colonization by pathogens, and may suppress *Campylobacter* colonization (Di Marcantonio et al., 2022).

Campylobacteriosis is the most commonly reported zoonosis in the European Union (EU), where broiler products are a common source of infection due to insufficient heat treatment or cross-contamination. According to the European Food Safety Authority (EFSA), 58% of human *Campylobacter jejuni* (*C. jejuni*) infections are associated with broiler meat (EFSA, 2020). Poultry feed with low pH and a high number of lactic acid bacteria (LAB) has been shown to reduce the susceptibility to *Campylobacter* colonization in broilers (Heres et al., 2003). This effect might be explained by the principles of pathogen inhibition mentioned above.

Although the prevalence of *Campylobacter* in conventional broiler production in Sweden is currently low, the problem remains in organic production. In 2021, 5% of tested conventional batches were *Campylobacter* positive at slaughter, whereas in organic production, 33% of tested flocks were positive (Swedish Poultry Meat Association, 2021). The higher frequency in the latter is due to the access to outdoor reservoirs of *Campylobacter*, as all organic poultry in the EU must have the opportunity to spend time outdoors (Commission Regulation (EC) 889/2008, 2008). In addition, organic poultry must be provided daily access to forage where silage is provided at some organic broiler farms (Crawley, 2015).

This study aimed to investigate the effects of daily intake of *Lactiplantibacillus plantarum* strain 256 (*L. plantarum* 256; *LP*256) on *C. jejuni* load in broiler's cecum and feces, together with the changes in their gut microbiota. In organic farming, silage can be supplied as forage to the broilers, and therefore we assessed the efficiency of providing *LP*256 both via silage inoculated with the strain and via direct supplementation in the drinking water. Concurrently, impact of haylage as a forage similar to silage but without any inoculum was tested. The effects of the treatments were evaluated on slow-growing (birds used in organic production) and fast-growing (conventional production) broilers in two separate trials under *C. jejuni* challenge.

2. Materials and methods

2.1. Experimental design and housing

The experimental setting was previously described in detail by Valečková et al. (2020); a brief description follows. Two experiments were conducted concurrently at the Swedish Livestock Research Centre of the Swedish University of Agricultural Sciences, with approval from the Uppsala region's animal ethics committee (approval number 5.8.18-16271/2017). The experiments were conducted using fast-growing broilers Ross 308 (R-308), used in conventional production in Sweden, and the Rowan Ranger broilers (RR), with a slower growth preferred in, e.g., organic broiler production. In Experiment 1, a total of 160 unsexed day-old R-308 broiler chickens were used for the 42-day (6-week) experiment, which is considered a normal period of growth for fast-growing strains in the EU. In Experiment 2 a total of 160 unsexed day-old RR broiler chickens (also referred to as "slow-growing broiler") were used in the 63-d experiment (9 weeks) corresponding to the age at which this broiler type is generally slaughtered in organic production systems in Sweden. In each study, broilers were randomly distributed in groups of eight individuals in 20 raised pens with four dietary treatments and five pen replicates for each treatment, arranged in a randomized block design. In both studies, two random broilers per pen were chosen as focal birds, representatives of the entire pen population. Focals were later on used for the collection of fecal droppings for *C. jejuni* quantification by agar plate culture and at the end of the experiment for cecal content sampling for a quantitative real-time polymerase chain reaction (qPCR) assay to quantify *C. jejuni* loads and for microbiota analysis done by 16S rRNA sequencing.

The experiments were performed in parallel during the winter in an insulated stable equipped with the facilities for automatic control of temperature and light. Each pen had a floor covered by fresh wood shavings and was equipped with a metal feeder and a 3-liter bell drinker.

2.2. Experimental diets

Detailed diet specification and forage preservation are stated in Valečková et al. (2020). In brief, fresh feed and water (including

treatments) were provided directly after the broilers' arrival and supplied daily. All experimental diets were based on organic compound feed (13 MJ/kg metabolizable energy and 230 g/kg DM crude protein) and the daily requirement of pellets in all treatment groups was estimated (based on production performance objectives) to ensure *ad libitum* provision. Broilers were assigned to four different treatment groups: silage, haylage, *LP*256, or control. Haylage treatment was included in the study as a forage similar to silage but without any inoculum. Silage and haylage experimental diets were composed as total mixed rations (TMR) containing 85% of pellets and 15% of respective forage (on a DM basis). Additionally, the *LP*256 and the control groups received the organic pelleted compound feed (no forage provided). The *LP*256 group had drinking water inoculated with *L. plantarum* 256 (10^7 CFU/mL).

Second-cut grass, with a seeding composition of 70% timothy and 30% meadow fescue, was used for the production of forages. Silage was inoculated with *L. plantarum* strain 256 during baling, providing an inoculum concentration of 10^8 CFU per gram fresh matter. Haylage bales were made without inoculum. After 3 months of storage, bales were separately opened, chopped and thereafter ground to 0.5–1 cm particles. Forage was afterward vacuum-packed (1 kg per bag) and bags were stored at a temperature below 0°C to maintain a similar feed quality throughout the experiments. Enumeration of epiphytic LAB on silage was performed in duplicates monthly (January, February, and March) during the trial period and the pH of silage juice was measured prior to the trials.

2.3. Bacterial strains and culturing conditions

Bacterial strains used in this study include *L. plantarum* 256 and *Campylobacter jejuni* #65. The *L. plantarum* strain (also known as *L. plantarum* NC7, Cosby et al., 1989) was previously used in our *in vitro* experiments and proved among other LABs to elicit the best inhibitory effect against *C. jejuni* #65 (unpublished data). The *L. plantarum* strain 256 isolate was stored at – 80°C in Luria Bertani (LB) broth with 20% glycerol. It was propagated in De Man, Rogosa and Sharpe broth for 24 h at 37°C for silage preparation and as a prophylactic probiotic in the study. *C. jejuni* #65 (ST-104, in ST-21 CC; isolated from a broiler chicken in the UK 2006) was cultured in Brucella broth at 42°C under microaerobic (85% N2, 10% CO2, 5% O2) conditions for 24 h. After 24 h incubation, optical density at 405 nm and plate counts were used to determine the infection dose used in the *C. jejuni* challenge.

2.4. *Campylobacter jejuni* colonization and quantification

To investigate the effects of the *L. plantarum* treatments on *C. jejuni* colonization of the broilers' ceca, all birds were orally challenged (also referred to as "infected") at 22 d of age in Experiment 1 and 29 d of age (corresponding to the 4 weeks of age at which organic broilers in Sweden must have access to an outdoor environment) in Experiment 2 (Figure 1). On the day of the challenge, 0.5 L of water with 10^6 CFU/mL of the *C. jejuni* strain #65 was provided in the bell drinker of each pen. The inoculated water was administered for 3 h

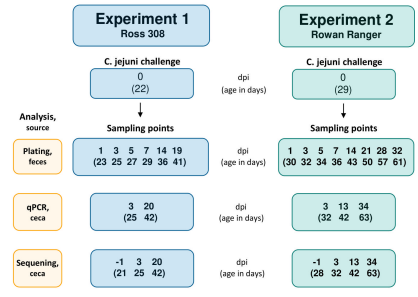

FIGURE 1
Flowchart with the *Campylobacter jejuni* challenge occasion and following sampling points in Experiment 1 (R-308) and Experiment 2 (RR). The challenge occasion and sampling points are presented as days post-infection (dpi), with the corresponding age of the birds (in days) indicated in brackets.

and *C. jejuni* viability in the water was determined by colony counts on blood agar plates at the start and end of the challenge.

The colonization pattern of *C. jejuni* was monitored during 19 days and 32 days for R-308 and RR, respectively, by fecal culture and colony counts on modified Charcole Cefoperazone Deoxycholate (mCCDA) agar plates. For fresh fecal sampling, two focal birds from each pen were placed individually in plastic boxes. Sterile plastic loops were used for the collection of droppings from the box bottom. Fecal samples were collected from all pens 1 day before the challenge with *C. jejuni*, to verify that the broilers were *Campylobacter* negative before the challenge. One hundred mg of fresh fecal droppings from the focal birds in each pen were collected in 1 mL LB medium supplemented with 20% glycerol on 1, 3, 5, 7, 14, and 19 days post-infection (dpi) in Experiment 1 and 1, 3, 5, 7, 14, 21, 28, and 32 dpi in Experiment 2 (Figure 1). Tubes were directly transported on ice to the laboratory for analysis. Samples were vortexed and centrifuged (100 × *g* for 15 s) to pellet crude fecal matter. Next, 100 µL was withdrawn and serially diluted in 10-fold dilution series. Afterward, 100 µL was plated on mCCDA and incubated for 26 h at 42°C under microaerobic conditions (Campygen, Thermo Fisher). After incubation, colonies were counted on the plate corresponding to the dilution that gave approximately 100 CFU per plate. Raw plate counts data are provided in Supplementary Figure 1 (Experiment 1) and Supplementary Figure 2 (Experiment 2).

2.5. Cecal samples collection

In both experiments, one random bird per cage was sacrificed 1 day before infection (−1 dpi) and 3 days after infection (3 dpi) by an intravenous injection of sodium pentobarbital through the wing vein; the birds age corresponding to mentioned dpi is stated in Figure 1. The cecal content was sampled with an aseptic procedure into 2.0 mL screw cap microtubes (Sarstedt AG & Co, Germany) and placed in liquid nitrogen (followed by storing at – 80°C until analyzed). At 42 days of age, all focal birds in Experiment 1 (20 dpi), and one random bird in each replicate in Experiment 2 (13 dpi) were sacrificed and sampled. Experiment 2 focal

birds were sacrificed at 63 days of age (34 dpi) followed by cecal sampling as described above. Samples were analyzed by a qPCR assay to assess *C. jejuni* colonization and the microbial composition of cecal content was investigated by 16S rRNA amplicon sequencing.

2.6. DNA extraction and qPCR-based *Campylobacter jejuni* quantification

For quantification of *C. jejuni* load in the broiler cecum using qPCR, a standard curve was developed as a reference for the proceeding analysis (Supplementary Table 1). Bacterial colonies of *C. jejuni #65* from a 36-h incubated mCCDA culture were suspended in 300 µL of phosphate-buffered saline (PBS). The suspension was briefly vortexed and divided into 100 µL duplicates, and was diluted in a 10-fold series of PBS for CFU counting on mCCDA plates and incubated under microaerobic conditions for 24 h at 42°C.

Simultaneously, another 100 µL replicate was extracted for DNA and qPCR analysis. The sample was mixed with 200 mg of 0.1 mm zirconia/silica beads (Biospec products, Bartlesville, USA) and 900 µL of ASL lysis buffer (Qiagen, Germany), briefly vortexed, and incubated at 95°C for 5 min to lyse cells, followed by immediate placement on ice for 10 min. The sample was then bead-beaten on Precellys24 sample homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 8000 rpm for 2 × 60 s with 30 s pause to disrupt bacterial cell walls mechanically. Centrifugation of the sample at 2500 x *g* for 1 min followed, and 200 µL of the supernatant was withdrawn into the sample tube together with 20 µL of proteinase K for DNA extraction. Extraction was performed on an EZ1 Advanced XL instrument (Qiagen, Germany) according to the manufacturer's instructions. The extract was diluted in a 10-fold series of nuclease-free water and used as a template in the real-time PCR for generating a standard curve.

A real-time PCR targeting the d65_1178 gene, specific to *C. jejuni* Strain #65 and its ST type ST-104 (ST-21 CC) was conducted using a primer pair adapted from Atterby et al. (2018). The PCR was performed on a CFX96 Optics Module C1000 Touch Thermal Cycler (Bio-Rad Laboratories, USA). The reaction mixture contained: 1 x SsoAdvanced Universal SYBR Green Supermix, 0.3 µL of forward and reverse primer each, and 1 µL of the template. Reactions were run in triplicates. The amplification parameters were as follows; 98°C for 3 min, 40 cycles of 98°C for 15 s and 63°C for 60 s and followed by a melt curve ranging from 65 to 95°C as a check for assay specificity. Generated qPCR data was analyzed on Bio-Rad CFX Manager 3.1 software (Bio-Rad Laboratories, USA) and Microsoft Excel. Raw qPCR data are provided in Supplementary Figure 3 (Experiment 1) and Supplementary Figure 4 (Experiment 2). The amplification efficiency of the PCR reaction was 76% with R^2 of 0.9996.

All the cecal samples followed the same pre-treatment, DNA extraction procedure as the *C. jejuni #65* suspension, with minor pre-treatment modifications. Modifications included; 400 µL of ASL lysis buffer, added to the sample and vortexed briefly to homogenize. Then, 120 µL of the sample was used in downstream steps. Sample DNA extracts were analyzed by qPCR and sequencing. For quantification, DNA extracts were run on qPCR along with a standard (*C. jejuni #65*) DNA extract. The CT values obtained from sample runs were compared to that of the standard and transformed into CFU using the generated standard curve equation. The generated CFU was multiplied by five to compensate for a five times dilution of the sample performed during pre-treatment, a dilution not performed on the standard suspension. The ultimate quantification was expressed in CFU/ml by multiplication of 10.

2.7. 16S rRNA sequencing

One hundred forty cecal sample DNA extracts were sequenced using the Illumina Miseq PE 250 sequencing platform at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). The 16S rRNA gene V3–V4 regions were amplified using Illumina primer set 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTAT CTAAT) with a barcode. All template DNAs were normalized to the same concentration. PCR reactions were performed with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA). PCR products were separated by electrophoresis on 2% agarose gel, purified with a Qiagen Gel Extraction Kit (Qiagen, Germany) and pooled at equal concentrations. Sequencing libraries were generated using NEBNext Ultra DNA Library Prep Kit (Illumina, USA) following the manufacturer's recommendations, and index codes were added. Library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA) and the Bioanalyzer 2100 system (Agilent Technologies, USA).

2.8. Sequence analysis

The raw sequencing data were uploaded to the National Center for Biotechnology Information database (NCBI) with accession number PRJNA876811. The bioinformatics data processing was performed by Quantitative Insights into Microbial Ecology 2 – QIIME2 (version 2020.2.0) (Bolyen et al., 2019). The barcode and primer sequence of raw demultiplexed reads were trimmed off and further processed by DADA2 to denoise and dereplicate reads, merge pair-end reads and remove chimeras (Callahan et al., 2016). The truncation length of 221 bp was used for both forward and reverse reads. The phylogenetic tree was built using FastTree and MAFFT alignment (Katoh et al., 2002; Price et al., 2010). The SILVA SSU Ref NR 99132 dataset was first trimmed to the corresponding primer region and trained as a classify-sklearn taxonomy classifier (Pedregosa et al., 2012; Quast et al., 2013; Bokulich et al., 2018). Subsequently, the amplicon sequence variants (ASV) were assigned taxonomy using the resulting classifier. After trimming and quality filtering, the sequencing of 16S rRNA gene yielded a total of 4,539,867 sequences from 140 samples. The ASV table was rarefied according to the minimum reads per sample (i.e., 21,377 reads) (Weiss et al., 2017). The generalized UniFrac distance matrix (alpha = 0.5) and alpha rarefaction was generated using the QIIME2 diversity plugin (Chen et al., 2012; Bolyen et al., 2019).

2.9. Statistical analysis

The data generated from plate counts and qPCR from both experiments were organized in Microsoft Corporation (2018) and statistically analyzed in SAS version 9.4 (SAS Institute Inc, 2013). Plating data are graphically represented as scatter panel plots showing bacterial counts as log (CFU/ml) and qPCR data as a box plot; plots were generated with R and the package ggplot2 (Wickham, 2016; R

Core Team, 2021). Data from Experiments 1 and 2 were treated by the same pattern.

Statistical analyses of fecal plate count data were performed with a mixed effect linear model (Proc Mixed procedure in SAS) due to the repeated measure structure of the data. The model included treatment, days post-infection (dpi), and their interactions as fixed factors and pen as a random factor. To account for the repeated structure when several observations were made on the same birds (focals) at different dpi we included an error term with an unstructured covariance matrix. Post-hoc tests were conducted to compare *C. jejuni* load (log CFU/ml) at individual dpi among treatment groups as well as to compare all dpi within each treatment. In order to compare *C. jejuni* loads through the whole challenge period between four treatment groups, plate count data were expressed as the mean of all observed dpi samples within one treatment (colonization mean). qPCR data were analyzed with the same mixed-effect linear model and in the same pattern as the plating data. However, the pen as a random factor and repeated structure were removed from the model since only one cecal sample per pen was analyzed. Residual plots were inspected to ensure that residual were approximately normally distributed with equal variances for all models. Results are considered significant if $p < 0.05$.

For cecal microbiota, diversity analyses were performed with the q2-diversity plugin. The rarefied ASV table was used to calculate the number of observed ASV. Kruskal–Wallis rank test with Benjamini & Hochberg (B-H) correction was used to observe statistical differences in a number of observed ASV between groups (i.e., dpi and treatment, Kruskal and Wallis, 1952; Benjamini and Hochberg, 1995). Principal coordinate analysis was used to visualize the difference in the microbial composition based on the generalized UniFrac distances. Permutational multivariate analysis of variance (PERMANOVA) test of generalized UniFrac distance matrix with (B-H) correction was conducted to evaluate the difference among groups (Anderson, 2001). To identify bacterial taxa that differed in abundance between groups, we performed an analysis of composition of microbiomes (ANCOM) (Mandal et al., 2015).

3. Results

3.1. Higher LAB concentration and lower pH in silage than in haylage

Monthly enumeration of LAB during the experiment period revealed that silage contained 8.0, 7.4, and 7.2 log CFU/g of LAB, respectively, while haylage levels were 5.0, 3.8, and 3.0 log CFU/g. Consequently, silage contained ≥3 × 10-log (CFU/g) higher LAB concentrations than haylage and a gradual decrease in LAB concentrations was observed in both matters. The silage pH measurement just prior to the experiment showed pH 4.4 while haylage displayed pH 6.2.

3.2. *Campylobacter jejuni* colonization impacted by silage and haylage treatment in R-308 but not in RR as determined by culture, no significant treatment effects determined by qPCR

Culture results revealed *Campylobacter jejuni* negativity in all birds prior to the infection and successful *C. jejuni* colonization in both broiler types after the challenge. In both experiments, *C. jejuni* loads peaked within 3 days after the challenge and thereafter colonization intensity had decreasing tendency with time.

In R-308, there was an overall significant treatment effect ($p = 0.023$) observed. Specifically, a significantly lower *C. jejuni* colonization mean was observed in the silage ($p = 0.010$) and haylage ($p = 0.013$) groups in comparison to the control (Figure 2). At 1 dpi, colonization in the silage group was significantly lower (2.01 logs) in comparison to the control group ($p = 0.039$). However, at the end of the experiment (19 dpi), there was no significant difference in the colonization between any of the treatments. No significant effect of *LP*256 (directly provided via the drinking water) treatment on *C. jejuni* loads was observed. A comparison of *C. jejuni* colonization within each treatment at 1 and 19 dpi (start and end of colonization period) revealed no significant difference in bacterial load (CFU/ml). The same was true for colonization comparison between the 3 dpi (supposed peak of *C. jejuni* load) and 19 dpi.

As determined by qPCR (Figure 3), *C. jejuni* loads in ceca at 3 dpi (25 days of age) or 20 dpi (42 days of age) were not significantly affected by dietary treatments in R-308. A comparison of *C. jejuni* colonization within treatment revealed a decreasing pattern between 3 dpi and 20 dpi. However, the differences in *C. jejuni* CFU/ml were not statistically significant.

No significant effect of treatments on *C. jejuni* loads was observed in RR as determined by culture (Figure 4). However, colonization comparison between the start of the infection period (1 dpi) and end of the trial (32 dpi) within each treatment revealed significant changes with mean reductions in *C. jejuni* of 2.65 and 2.46 10-log (CFU/ml) for *LP*256 and haylage, respectively ($p = 0.006$ and $p = 0.017$, respectively). Colonization comparison between the supposed peak of bacterial load (3 dpi) and end of the trial (32 dpi) displayed significant *C. jejuni* reduction in all treatment groups; $p = 0.002$, $p = 0.001$, $p = 0.001$, and $p = 0.001$ for control, *LP*256, haylage and silage group, respectively.

At 3, 13, or 34 dpi, no significant treatment effects on the ceca *C. jejuni* loads were observed in RR as determined by qPRC (Figure 5). Comparing *C. jejuni* loads between the supposed peak of bacterial colonization (3 dpi; 34 days of age) and end of the trial (34 dpi; 63 days of age) revealed significant reductions in control, *LP*256, and haylage group ($p = 0.001$, $p = 0.001$, and $p = 0.024$, respectively). A comparison between cecal *C. jejuni* colonization at 13 dpi (42 days of age) and 34 dpi for each treatment displayed significant *C. jejuni* reductions in the control, *LP*256, and silage groups ($p = 0.001$, $p = 0.032$, and $p = 0.014$, respectively).

3.3. *Firmicutes* and *Bacteroidota* dominated both R-308 and RR cecal microbiota, with significant changes observed in their relative abundances after the *Campylobacter* challenge

Characterization of the cecal microbiota composition before and after the *C. jejuni* challenge in Experiments 1 and 2 was performed by 16S rRNA amplicon sequencing. A total of 140 samples were analyzed and altogether 675 amplicon sequence variants (ASVs) were identified, representing 122 taxonomic genera, 52 families, 33 orders, 13 classes, and 5 phyla. The rarefaction curves of observed ASVs revealed sufficient sequencing depth to capture species richness at all time points tested in Experiment 1 (Supplementary Figure 5) and Experiment 2 (Supplementary Figure 6).

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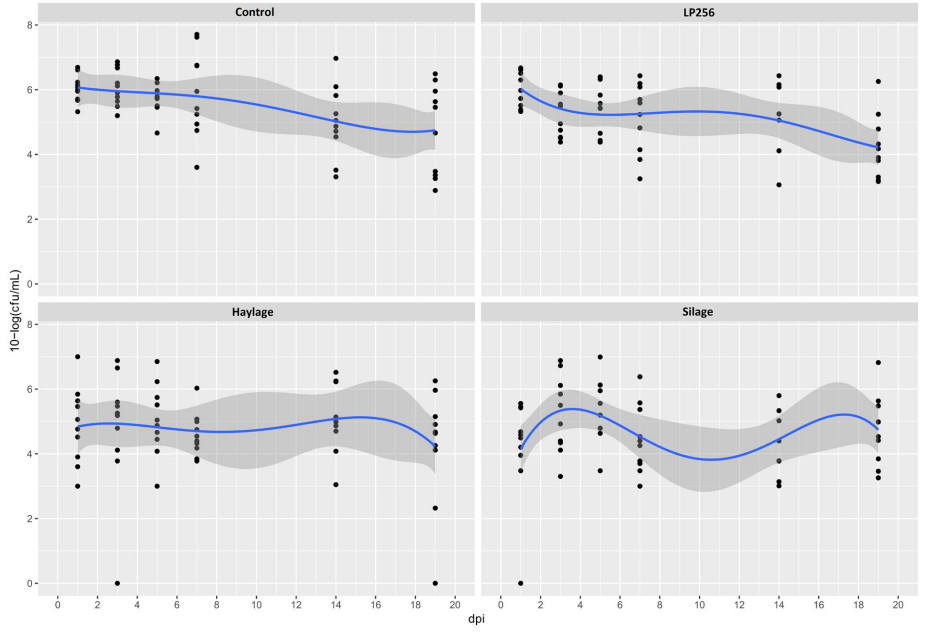

FIGURE 2
Culture-based colonization patterns of *C. jejuni* in four dietary treatment groups in Experiment 1. Black dots (data points) represent 10-log (CFU/ml) in individual fecal samples at a given day post-infection (dpi). The blue line is a smooth curve representing the trend of colonization based on the mean 10-log (CFU/ml) in each treatment with a 95% confidence band.

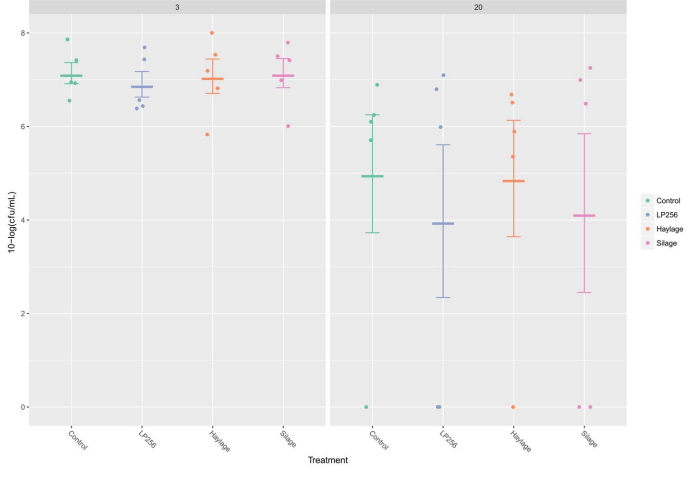

FIGURE 3
Quantitative PCR-based colonization dynamics of *C. jejuni* in the four dietary treatment groups at 3 and 20 days post-infection (dpi) in Experiment 1. The dots represent *C. jejuni* load (10-log CFU/ml) in individual caecal samples and line bars represent the mean 10-log CFU/ml with standard deviation.

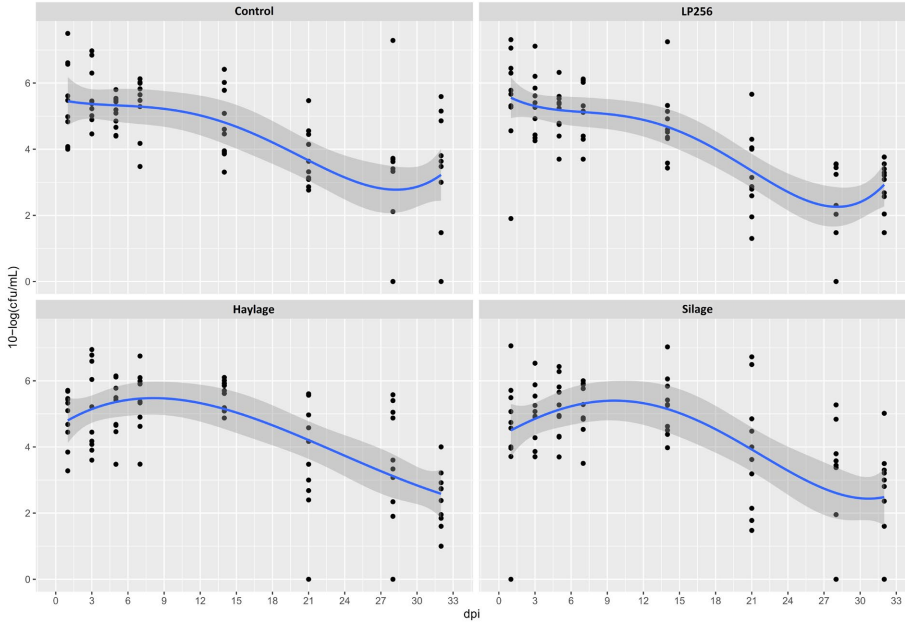

FIGURE 4
Culture-based colonization patterns of *C. jejuni* in four dietary treatment groups in Experiment 2. Black dots (data points) represent 10-log (CFU/ml) in individual fecal samples at a given day post-infection (dpi). The blue line is a smooth curve representing the trend of colonization based on the mean 10-log (CFU/ml) in each treatment with a 95% confidence band.

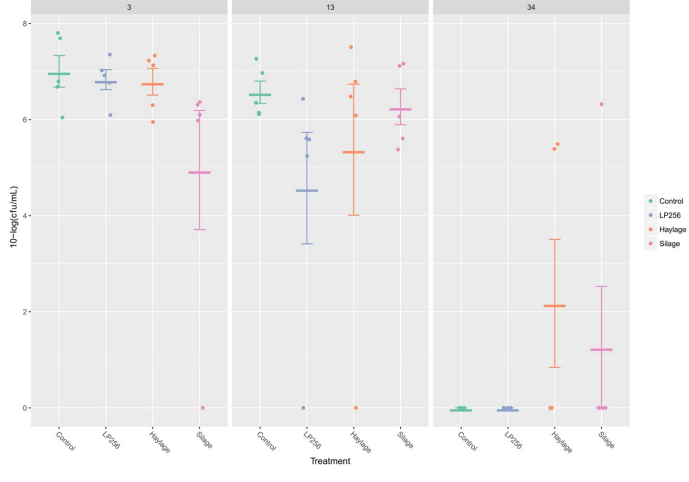

FIGURE 5
Quantitative PCR- based colonization dynamics of *C. jejuni* in the four dietary treatment groups at 3, 13, and 34 days post-infection (dpi) in Experiment 2. The dots represent *C. jejuni* load (10-log CFU/ml) in individual caecal samples and line bars represent the mean 10-log CFU/ml with standard deviation.

In detail, 619 ASVs, representing 109 taxonomic genera and 5 phyla were observed in Experiment 1. Results of the principal coordinate analysis (PCoA) are shown in Figure 6 to visualize the variation of cecal microbiota between different days post-infection. Significant differences in the gut microbiota composition between different dpi were observed ($p = 0.001$), while no clear effect of the feed treatments on cecal microbiota composition was found; therefore, the treatments were pooled for further analysis.

At the phylum level, *Firmicutes* and *Bacteroidota* comprised more than 97.5% of the bacteria's relative abundance (RA) in all treatment groups at −1, 3, and 20 dpi, suggesting that they were the major components of the cecal microbiota (Figure 7). Since the different dietary treatments had no influence on the gut microbiota composition, treatments were pooled together and changes between different days post-infection were investigated. Changes in the RA at phylum level was observed after the *Campylobacter* challenge; a temporary decrease in the RA of phylum *Firmicutes* (mean RA at −1, 3, and 20 dpi was 84.2, 74.7, and 85.4% respectively) was substituted by a corresponding significant increase in *Bacteroidota* (mean RA at −1, 3, and 20 dpi was 14.5, 23.7, and 13.4%, respectively). The RA of phylum *Proteobacteria* decreased at 3 dpi and returned to a similar level as before the *C. jejuni* challenge at 20 dpi (mean RA at −1, 3, and 20 dpi was 1.3, 0.7, and 1.1% respectively). A significant increase in the RA of phyla *Campilobacterota* was observed at 3 dpi (mean RA of *Campilobacterota* at −1, 3, and 20 dpi was 0.01, 0.9, and 0.1% respectively). The RA of *Actinobacteriota* ranged from 0.02 to 0.04%.

At the genus level, the top 25 genera (Table 1) constituted 89% of the total sequencing read pool. Genus *Campylobacter* was added as the 26th genus due to the interest of this study. A description of major changes in the RA at the genus level between different dpi follows: genus *Bacteroides* was the most dominant in the cecal microbiota at −1 dpi and clearly most dominant after the *C. jejuni* challenge (3 dpi). Thereafter, a considerable decrease was observed to the advantage of other genera at 20 dpi (Table 1). *Clostridia* UCG-014 and uncultured *Ruminococcaceae* continuously increased in RA throughout the sampling points; where *Clostridia* UCG-014 became the most abundant genus at 20 dpi. A significant decrease in the RA of the twelfth most abundant genus *Lactobacillus* was observed after the *C. jejuni* challenge at 3 dpi. However, at 20 dpi the RA of this genus reached higher levels than before *C. jejuni* challenge. In the genera *Clostridia* vadinBB60 group and unclassified *Lachnospiraceae*, a decrease in RA appeared at 3 dpi and remained at a similar level at 20 dpi. The RA of the genera *Faecalibacterium* (the second most dominant bacterial genus in broilers' ceca), *Eisenbergiella*, *Subdoligranulum*, and *Escherichia-Shigella* decreased after *C. jejuni* challenge (3 dpi) but eventually, an increase was observed in all four genera at 20 dpi. As expected, the RA of genus *Campylobacter* significantly increased after the challenge (3 dpi) but had diminished at 20 dpi. Although clear general trends in the mean RA of different genera could be observed over the experiment as described above, there were high individual variations in the birds' cecal microbiota composition within the same treatment group at each infection time point (dpi).

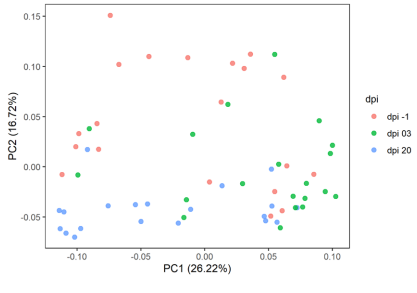

FIGURE 6
Principal coordinate analysis (PCoA) plot showing the differences in beta diversity based on generalized UniFrac distances between samples at −1, 3, and 20 days post-infection (dpi); represented by 20 caecal samples, respectively, for R-308. The dpi −1 is represented by samples before infection.

In Experiment 2, sequencing results comprised 671 ASVs, representing 121 taxonomic genera and 5 phyla. The results of the principal coordinate analysis are presented in the PCoA plot (Figure 8), where significant differences in the microbiota composition were observed ($p = 0.001$), while no clear effect of the feed treatments was found.

As seen in Experiment 1, cecal microbiota composition at the phylum level was dominated by *Firmicutes* and *Bacteroidota* also in Experiment 2; representing together at least 92.2% of the bacteria in all treatment groups (Figure 9) at −1, 3, 13, and 34 dpi. Proportional changes in RA were observed after the *C. jejuni* challenge (dpi 3) by an increase of phylum *Firmicutes* (mean RA at −1, 3, 13, and 34 dpi was 70.6, 84.5, 80.1, and 81.8% respectively) with a concomitant decrease of the taxonomic group *Bacteroidota* (mean RA at −1, 3, 13, and 34 dpi was 26.9, 13.6, 18.5, and 17.0% respectively). This was in contrast to the reverse pattern observed in Experiment 1. The RA of phylum *Proteobacteria* decreased after the infection (3 dpi), remained on a similar level at 13 dpi, and eventually increased again at 34 dpi; mean RA at −1, 3, 13, and 34 dpi was 2.4, 1.6, 1.3, and 2.1%, respectively. A significant increase in RA of the phylum *Campilobacterota* was observed at 3 dpi; mean RA at −1, 3, 13, and 34 dpi was 0.01, 0.3, 0.1, and 0.01%, respectively. The RA of phylum *Actinobacteriota* increased continuously throughout the experiment, ranging from 0.03–0.09%.

The top 25 genera (Table 2) constituted 91% of the total sequencing read pool. Genus *Campylobacter* was added as the 26th genus due to the interest of the study. A description of the trends in relative abundance at genus level during the experiment period follows: *Faecalibacterium* was the second most abundant genus at −1 dpi, the most abundant after the *C. jejuni* challenge (3 dpi), and clearly the most abundant at 13 and 34 dpi. *Bacteroides* dominated the cecal microbiota at −1 dpi. Despite a considerable decrease after *C. jejuni* challenge, the genus was the second most dominant at 3, 13, and 34 dpi. The RA of the genera *Clostridia* UCG-014, *Ruminococcus torques* group, and uncultured *Ruminococcaceae* peaked at 3 dpi, and thereafter gradually decreased at 13 and 34 dpi. Genus *Lactobacillus* was the ninth most abundant bacteria present in the ceca and its RA increased throughout all sampling points. Relative abundance of genera unclassified *Lachnospiraceae* and *Subdoligranulum* increased at 3 dpi, decreased at 13 dpi, and was maintained at a similar level at 34 dpi. In the genus *Clostridia* vadinBB60 group, a continuous decrease in RA was seen throughout the sampling points. *Escherichia-Shigella* decreased after the challenge and remained on the same level

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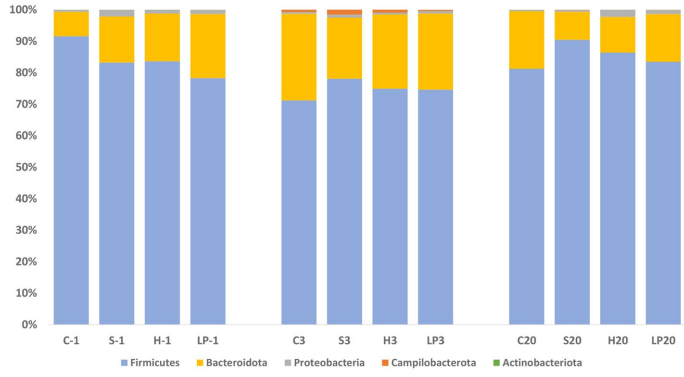

FIGURE 7
Phylum-level relative abundance (%) of broiler caecal microbiota in Experiment 1 (R-308) at each treatment and days post-infection (dpi): −1, 3, and 20. The treatment groups shown are the following: dietary treatment and dpi. C, control feed; S, diet based on 85% of pellets and 15% of silage; H, diet based on 85% of pellets and 15% of haylage; LP, control feed and water inoculated with 10^7 CFU/mL of viable *L. plantarum* 256.

TABLE 1 The mean and SD of relative abundance (%) of the top 25 genera of broiler cecal microbiota at each day post-infection (dpi) in Experiment 1.

Family	Genus	−1 dpi (%)	3 dpi (%)	20 dpi (%)
Bacteroidaceae	*Bacteroides*	14.5 ± 5.2	23.7 ± 3.3	13.4 ± 4.2
Ruminococcaceae	*Faecalibacterium*	9.4 ± 4.0	8.2 ± 3.9	13.7 ± 1.5
Same as genus	*Clostridia* UCG-014	4.1 ± 0.7	9.9 ± 1.9	16.1 ± 3.4
Same as genus	*Clostridia* vadinBB60 group	12.7 ± 5.3	6.8 ± 2.7	6.4 ± 0.6
Lachnospiraceae	unclassified *Lachnospiraceae*	8.2 ± 1.6	6.9 ± 0.5	6.3 ± 0.9
Lachnospiraceae	*Ruminococcus torques* group	6.2 ± 0.9	6.7 ± 1.4	5.4 ± 0.9
Lachnospiraceae	*Eisenbergiella*	3.7 ± 0.9	2.6 ± 0.3	2.7 ± 0.6
Ruminococcaceae	*Subdoligranulum*	2.0 ± 1.3	1.8 ± 0.7	3.5 ± 1.1
Ruminococcaceae	*Negativibacillus*	2.4 ± 0.2	2.0 ± 1.2	2.1 ± 0.2
Oscillospiraceae	*Colidextribacter*	2.2 ± 0.3	2.5 ± 0.5	1.6 ± 0.2
Ruminococcaceae	uncultured *Ruminococcaceae*	1.6 ± 0.1	1.8 ± 0.2	2.2 ± 0.4
Lactobacillaceae	*Lactobacillus*	1.7 ± 0.7	0.9 ± 0.9	2.9 ± 1.2
Oscillospiraceae	uncultured *Oscillospiraceae*	1.3 ± 0.3	2.1 ± 0.4	1.8 ± 0.5
Lachnospiraceae	*Lachnospiraceae* NK4A136 group	2.6 ± 0.7	1.2 ± 0.2	1.4 ± 0.5
Peptostreptococcaceae	unclassified *Peptostreptococcaceae*	4.3 ± 7.6	0.1 ± 0.2	0.0 ± 0.0
Same as genus	[*Eubacterium*]_coprostanoligenes_group	1.4 ± 0.2	1.2 ± 0.1	1.6 ± 0.1
Lachnospiraceae	*Lachnospiraceae* GCA-900066575	1.4 ± 0.3	1.2 ± 0.4	1.1 ± 0.4
Oscillospiraceae	unclassified *Oscillospiraceae*	1.1 ± 0.3	1.9 ± 0.7	0.5 ± 0.3
Oscillospiraceae	*Oscillibacter*	1.2 ± 0.3	1.2 ± 0.1	1.0 ± 0.2
Lachnospiraceae	*Blautia*	1.6 ± 0.5	1.0 ± 0.1	0.8 ± 0.2
Lachnospiraceae	*Lachnoclostridium*	1.5 ± 0.5	1.1 ± 0.2	0.8 ± 0.2
Ruminococcaceae	*Ruminococcaceae* Incertae Sedis	1.0 ± 0.3	1.1 ± 0.3	0.9 ± 0.1
Enterobacteriaceae	*Escherichia-Shigella*	1.2 ± 0.6	0.7 ± 0.2	1.1 ± 0.9
Same as genus	*Bacilli*_RF39	0.6 ± 0.3	1.0 ± 0.2	1.4 ± 0.4
Butyricicoccaceae	*Butyricicoccus*	0.8 ± 0.1	1.0 ± 0.3	0.5 ± 0.2
*Campylobacteraceae	**Campylobacter*	0.01 ± 0.01	0.9 ± 0.5	0.1 ± 0.1

Genus *Campylobacter* (*) was added as the 26th genus due to the interest of the study.

at 13 dpi; thereafter an increase was observed at the end of the trial. The RA of *Campylobacter* significantly increased after the infection (3 dpi) and declined at 13 and 34 dpi. As observed in Experiment 1, a high individual variation in birds' cecal microbiota composition was observed also in Experiment 2.

4. Discussion

Previous studies have shown that probiotics can modulate broilers' gastrointestinal microbiota, provide beneficial health effects, and increase birds' resistance to pathogens (Pourabedin and Zhao, 2015). In particular, LAB have been shown to provide inhibitory effects on *C. jejuni* colonization, and feed additives containing such bacteria could therefore be a promising approach to reduce the occurrence of *Campylobacter* spp. in primary production (Guyard-Nicodème et al., 2016). Although some studies have reported promising effects, there are challenges related to the storage, distribution, and rationally feasible means of administration of such probiotics to the broilers (Krysiak et al., 2021). In this study, we assessed the possibility of providing LAB by the inclusion of grass silage inoculated with the strain *Lactiplantibacillus plantarum* 256 in the broiler's daily feed. According to Commission Regulation (EC) 889/2008 (2008), all birds kept in organic settings in the EU must have daily access to forage, with grass silage being one of the allowable options. With this requirement in mind, we sought to investigate whether the silage may serve as a diet component with the potential to reduce *C. jejuni* in broilers' guts. In addition, to evaluate whether the potential effect was caused by *L. plantarum 256* itself, we also tested the direct provision of this strain via drinking water.

Our results from colony counts after agar plate culturing for R-308 (Experiment 1) displayed lower *C. jejuni* mean colonization in the silage and haylage treatment groups and significantly lower *C. jejuni* colonization (2 logs) in the silage group compared to the control 1 day after the challenge. However, this effect did not last and no differences in colonization were observed between treatment groups at the end of the experiment period. This result suggests that silage and to some extent haylage could have an inhibitory effect against low loads of ingested *C. jejuni* in the R-308, but clearly could not protect the broilers from *C. jejuni* colonization. A similar initial inhibitory effect could not be observed in the RR where no differences in *C. jejuni* loads between the treatment groups were observed in Experiment 2. High LAB content and low pH in fermented feeds have been previously reported as promising feed attributes in order to reduce the Ross 308 broiler chickens' susceptibility to *Campylobacter* spp. colonization (Heres et al., 2004). In the current study, lactic acid bacteria counts in ensiled forage and pH evaluation revealed that silage contained notably higher LAB concentrations and lower pH in comparison to the haylage. This may partly explain why *C. jejuni* loads at the beginning of the challenge in Experiment 1 were significantly lower in the silage group compared to the control, while only a moderate (non-significant) reduction was observed in the haylage treatment.

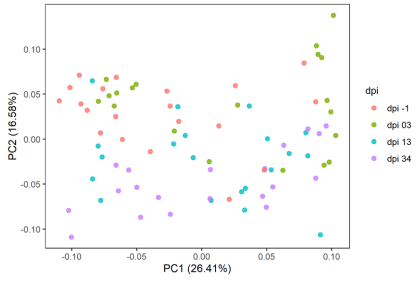

FIGURE 8
Principal coordinate analysis (PCoA) plot showing differences in beta diversity based on generalized UniFrac distances between samples at −1, 3, 13, and 34 days post-infection (dpi); represented by 20 samples, respectively, for RR. The dpi −1 is represented by samples before infection.

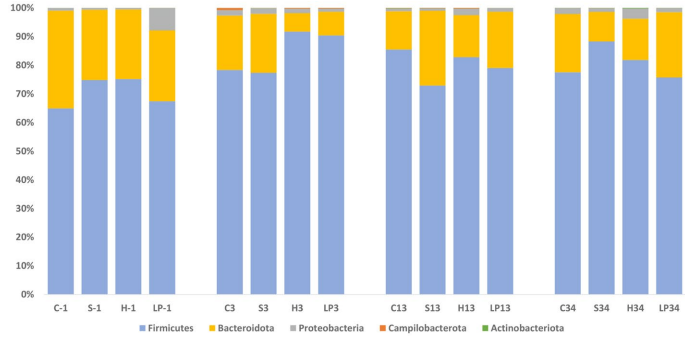

FIGURE 9
Phylum relative abundance (%) of broiler caecal microbiota in Experiment 2 (RR) at each treatment and days post-infection (dpi): −1, 3, 13, and 34. The treatment groups shown are the following: dietary treatment and dpi. C, control feed; S, diet based on 85% of pellets and 15% of silage; H, diet based on 85% of pellets and 15% of haylage; LP, control feed and water inoculated with 10^7 CFU/mL of viable *L. plantarum* 256.

TABLE 2 The mean and SD of relative abundance (%) of the top 25 genera of broiler cecal microbiota at each day post-infection (dpi) in Experiment 2.

Family	Genus	−1 dpi (%)	3 dpi (%)	13 dpi (%)	34 dpi (%)
Ruminococcaceae	*Faecalibacterium*	14.1 ± 4.8	14.7 ± 2.4	25.9 ± 4.2	27.9 ± 4.6
Bacteroidaceae	*Bacteroides*	25.9 ± 5.8	14.6 ± 8.7	18.5 ± 5.8	17.0 ± 5.7
Same as genus	*Clostridia* UCG-014	6.9 ± 0.9	13.9 ± 3.2	9.5 ± 1.7	7.2 ± 2.6
Lachnospiraceae	unclassified *Lachnospiraceae*	5.9 ± 1.8	6.3 ± 0.4	5.0 ± 0.8	5.6 ± 0.7
Ruminococcaceae	*Subdoligranulum*	4.2 ± 1.4	6.1 ± 3.7	2.7 ± 0.7	3.0 ± 1.6
Same as genus	*Clostridia* vadinBB60 group	5.3 ± 1.8	5.0 ± 2.1	3.3 ± 0.6	2.1 ± 1.1
Lachnospiraceae	*Ruminococcus torques* group	4.1 ± 0.2	4.4 ± 0.6	3.6 ± 0.4	3.2 ± 0.7
Lachnospiraceae	*Eisenbergiella*	2.5 ± 0.6	2.1 ± 0.2	2.9 ± 0.9	2.8 ± 0.8
Lactobacillaceae	*Lactobacillus*	1.1 ± 0.5	2.0 ± 0.6	2.6 ± 0.4	3.1 ± 1.1
Ruminococcaceae	*Negativibacillus*	2.3 ± 0.1	1.8 ± 0.2	2.1 ± 0.4	2.4 ± 0.6
Ruminococcaceae	uncultured *Ruminococcaceae*	2.0 ± 0.3	2.2 ± 0.5	2.0 ± 0.3	1.9 ± 0.5
Enterobacteriaceae	*Escherichia-Shigella*	2.8 ± 3.4	1.3 ± 0.6	1.3 ± 0.7	1.7 ± 1.0
Oscillospiraceae	*Colidextribacter*	2.0 ± 0.3	1.8 ± 0.3	1.6 ± 0.4	1.3 ± 0.2
Lachnospiraceae	*Lachnospiraceae* NK4A136 group	1.1 ± 0.9	0.9 ± 0.1	1.7 ± 0.7	2.6 ± 3.3
Same as genus	[*Eubacterium*] coprostanoligenes gr.	1.6 ± 0.2	2.3 ± 0.6	1.5 ± 0.3	0.9 ± 0.3
Oscillospiraceae	uncultured *Oscillospiraceae*	1.6 ± 0.3	1.5 ± 0.2	1.2 ± 0.3	1.3 ± 0.3
Oscillospiraceae	*Oscillibacter*	1.3 ± 0.3	1.2 ± 0.1	1.1 ± 0.3	1.4 ± 0.3
Same as genus	*Bacilli* RF39	1.0 ± 0.4	1.1 ± 0.5	1.2 ± 0.7	1.4 ± 0.6
Lachnospiraceae	*Lachnoclostridium*	1.1 ± 0.3	1.1 ± 0.2	0.8 ± 0.1	1.2 ± 0.4
Lachnospiraceae	*Lachnospiraceae* GCA-900066575	0.9 ± 0.3	0.8 ± 0.2	0.8 ± 0.2	1.1 ± 1.1
Oscillospiraceae	*Flavonifractor*	0.7 ± 0.1	0.9 ± 0.2	0.7 ± 0.2	1.1 ± 0.3
Erysipelatoclostridiaceae	*Erysipelatoclostridium*	0.6 ± 0.3	1.2 ± 1.0	0.7 ± 0.5	0.4 ± 0.3
Lachnospiraceae	*Blautia*	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.1	0.8 ± 0.1
Ruminococcaceae	*Ruminococcus*	0.4 ± 0.1	0.8 ± 0.4	1.2 ± 0.4	0.4 ± 0.2
Ruminococcaceae	*Ruminococcaceae* Incertae Sedis	0.8 ± 0.4	0.8 ± 0.2	0.6 ± 0.2	0.5 ± 0.2
*Campylobacteraceae	**Campylobacter*	0.01 ± 0.01	0.31 ± 0.29	0.13 ± 0.09	0.01 ± 0.01

Genus *Campylobacter* (*) was added as the 26th genus due to the interest of the study.

In vivo trials investigating the silage effect on *C. jejuni* colonization are currently rare. A study published by Ranjitkar et al. (2016) showed no significant differences in the load of *C. jejuni* between the treatment groups provided with different levels of crimped kernel maize silage in diets (replacement of 15 and 30% of pelleted feed with maize-silage) in comparison to the control. This discrepancy in results may be explained by differences in the type of silage, consumption levels, or by effects of broiler type and and age. The broiler type and age are also likely explanations as to why the reduction of *C. jejuni* loads were observed at the beginning of Experiment 1 in the R-308, while no effect was observed in the RR in Experiment 2. It is well known that fast-growing birds have a higher feed intake than slow-growing ones (Quentin et al., 2004; Sarica et al., 2020; Jong et al., 2021). For that reason, it was of interest to test the effects of silage in both fast- and slow-growing broilers. Hence, since a numerically higher intake of total mixed ratios, containing forage as silage and haylage, was observed in Ross 308 compared to Rowan Ranger birds (Valečková et al., 2020), it is possible that the inhibitory effect of silage on *C. jejuni* colonization seen at the beginning of Experiment 1 was related to the level of daily consumption of forage.

No significant treatment effect was seen on the reduction of *C. jejuni* in feces or ceca when *Lactiplantibacillus plantarum* 256 was directly provided in the drinking water to the R-308 or RR, neither by plate counts nor by qPCR. This suggests that although extracts from silage inoculated with this strain could inhibit *C. jejuni* growth *in vitro* (unpublished data), the presence of this strain in the broiler's intestinal tract at the level of administration (10^7 CFU/mL) used in this study could not effectively inhibit *C. jejuni* colonization. This could be related both to dosage and to the strain used. In a study by Arsi et al. (2015), 26 LAB isolates with the greatest inhibitory activity *in vitro* were further tested in a broiler trial where birds were challenged with 10^4 CFU *C. jejuni* in a 100 µL suspension of tryptone salt broth. Only 3 out of these 26 isolates demonstrated a reduction in *Campylobacter* counts (approximate 1–2 log) in comparison to the control. *In vitro* assay results are known to not always translate into comparable results under *in vivo* settings, due to differences in the final probiotic supplement composition, its dose and application pattern, trial conditions, and thus different outcomes of the probiotic activity. In addition, *in vitro* studies do not take into account the variability and complexity of the birds' gastrointestinal environment

and their interaction with probiotics and *Campylobacter* strains (Smialek et al., 2021). Nevertheless, inhibitory effects on *C. jejuni in vivo* after direct administration of *Lactiplantibacillus* spp. have been demonstrated in several studies. For example, Saint-Cyr et al. (2017) showed that Ross broilers treated with oral gavage of *Lactobacillus salivarius* SMXD51 (10^7 CFU) at 24 h after hatch displayed a significant reduction in *C. jejuni* loads present in the gut at 14 days of age (0.82 logs) and 35 days of age (2.81 logs) in comparison to the control. Additionally, Neal-McKinney et al. (2012) reported that *Lactobacillus crispatus* JCM 5810 administered to broiler chickens by oral gavage (10^8 CFU) at the day of hatch and 4 days post-hatch was an effective competitive exclusion organism for *C. jejuni* resulting in a reduction in the total number of *C. jejuni* colonized broilers and lower microbial load at 21 days post-hatch.

In both experiments in this study, *C. jejuni* loads in feces peaked at the beginning of the challenge, after which a decrease over time was observed. This is in agreement with previous findings, e.g., Achen et al. (1998) found that 70% of the broilers were shedding *C. jejuni* within 48 h after artificial infection, and a steady decline in fecal shedding was observed after the third-week post-infection; at 6 weeks after infection, only 38% of the birds were shedding *C. jejuni* in their feces. In the current study, the decline of *C. jejuni* loads with time was more prominent in Experiment 2 (in the RR), where the comparison between the peak of *C. jejuni* bacterial load at 3 dpi to that at the end of the challenge at 34 dpi, displayed significant *C. jejuni* reduction in all treatment groups. However, this was likely due to the fact that in Experiment 2, *C. jejuni* colonization after the challenge was monitored for 2 weeks longer than in Experiment 1.

Campylobacter jejuni loads in cecal samples from both experiments were quantified using a qPCR assay. Comparison between cecal samples analyzed by qPCR and fecal samples analyzed by plate counting revealed higher numbers of *C. jejuni* in cecal samples in both experiments, consistent with previous studies (Berrang et al., 2000; Rudi et al., 2004). However, in some cases, *C. jejuni* loads in cecal samples were below the detection limit of the qPCR, despite fecal cultures being *C. jejuni* positive. This inconsistency can be attributed to the lower sensitivity of our qPCR, whose limit of detection was 3.3×10^5 CFU/g (Appendix 3) and is likely the reason for the significant *C. jejuni* reduction observed in Experiment 2 when different infection time points (3 dpi to 34 dpi and 13 dpi to 34 dpi) were compared. Consistent with the findings from the *C. jejuni* colony counts from fecal samples (except observation in the forage treatments in Experiment 1), there were no significant effects of the dietary treatments on the cecal *C. jejuni* loads in either of the two experiments. Similarly, there was no significant reduction in cecal *C. jejuni* loads between 3 dpi and the end of Experiment 1, but a significant reduction was seen at the end of Experiment 2 in *LP*256, haylage, and silage groups. This reduction seemed to be independent of the treatment, consistent with our culture-based results from fecal samples.

The broiler digestive tract is colonized by a wide variety of bacterial species, with the caecum being by far the most densely colonized and studied microbial site of the gut (Pourabedin and Zhao, 2015). Generally, the major phylum in the broiler cecal microbiota is *Firmicutes*, followed by two less abundant phyla, *Bacteroidota*, and *Proteobacteria* (Oakley et al., 2014; Kers et al., 2018). This is reflected in our current results from Illumina 16S amplicon sequencing of cecal samples at different infection time points throughout the two experiments, where *Firmicutes* was the most abundant phylum, followed by the phylum *Bacteroidota*. Lower relative abundances of *Proteobacteria*, *Campilobacterota* (as a result of the *C. jejuni* challenge), and *Actinobacteriota* were observed. Before the *C. jejuni* challenge (−1 dpi), a lower relative abundance of *Bacteroidota* was observed in all treatment groups in R-308, than its RA in RR. Based on results from previous studies by Connerton et al. (2018) and Richards et al. (2019), this difference could be due to the type of broiler used, but also due to the different ages of the birds at the *C. jejuni* exposure since the RR birds in Experiment 2 were challenged 7 days later that the R-308 in Experiment 1. *Actinobacteria* and *Proteobacteria* together represented less than 2 and 2.5% in R-308 and RR cecal samples, respectively, which aligns with their usual representation of around 2 to 3% in the total broiler cecal microbiota (Rychlik, 2020).

There were no significant effects of the different feed treatments on the broiler's cecal microbiota composition in either of the two different broiler types. However, several distinct changes in relative abundance related to the *C. jejuni* challenge were noted both on phylum and genus levels. In Experiment 1, a decrease in the RA of *Firmicutes* appeared in the R-308 after the *C. jejuni* challenge, which was accompanied by a parallel increase in the RA of *Bacteroidota*. In Experiment 2, the opposite was observed. Previous studies have reported changes in *Bacteroidota* abundance linked to interactions with *Campylobacter*, where, e.g., elevated RA of *Bacteroidetes* was found in *Campylobacter-positive* broilers (Sofka et al., 2015), in agreement with our observations at 3 dpi in R-308. Interestingly, the opposite correlation was observed in a study by Sakaridis et al. (2018) where the elevated abundance of *Firmicutes* and decreased *Bacteroidetes* levels were found in broilers with high *Campylobacter* counts, in line with our observations in RR after the *C. jejuni* challenge. This shows the complexity of gut microbiota interactions, where one stimulus (*C. jejuni* challenge in this case) may have opposing effects on the microbiota composition in two different broiler types.

In both experiments, the predominant phylum *Firmicutes* largely consisted of class Clostridia represented by the genera *Faecalibacterium*, *Clostridia* UCG-014, *Clostridia* vadinBB60 group, and unclassified *Lachnospiraceae*, with other bacteria belonging to families *Lachnosporaceae* and *Ruminococcaceae* at lower percentages. Phylum *Bacteroidota* on the other hand consisted of the sole genus *Bacteroides*. This genus is able to produce short-chain fatty acids, compounds contributing to maintaining mucosal integrity, immunity, and health of broilers (Ali et al., 2022). The most dominant genus in the phylum *Proteobacteria* was the facultatively anaerobic *Escherichia-Shigella*, whose abundance decreased after the *C. jejuni* challenge. This was in contrast to the genus *Clostridia* UCG-014, whose relative abundance increased after the *C. jejuni* challenge. Similar findings were reported by Awad et al. (2016), where 14-day-old Ross 308 broilers were challenged with 1×10^8 CFU of *C. jejuni* NCTC 12744. In that study, *C. jejuni* colonization was associated with an alteration of the gut microbiota with infected birds having a significantly lower abundance of *Escherichia coli*, while the level of *Clostridium* spp. was higher in infected birds compared to non-infected. However, it should be noted that the higher abundance of *Clostridium spp*. induced after the *C. jejuni* challenge was not straightforward in the present study, since a

concomitant decrease in the relative abundance of the genus *Clostridia* vadinBB60 group was observed. Notably, 23 out of the top 25 genera were commonly observed in both R-308 and RR. In spite of this fact, high individual variation in the birds' cecal microbiota composition within the same broiler type, treatment group, and at the same infection time point in relation to the *C. jejuni* challenge was observed. This is in agreement with a previous study where great individual microbiota variation between animals within a single uniformly derived and treated group, under highly controlled experimental conditions, was reported (Stanley et al., 2014). The authors of that study speculate that the likely reasons for this variation are the lack of exposure to maternally obtained bacteria and the sensitivity to colonization by environmental bacteria in hatcheries.

Our aim with this study was to compare different strategies to administer LAB in order to increase their content in the broilers' gut and create an unfavorable environment for *Campylobacter*. The relative abundance of genus *Lactobacillus* in both Experiments was relatively high, the twelfth most abundant bacteria in the gut in Experiment 1 and the ninth most abundant in Experiment 2. However, neither the addition of silage LAB (including *Lactiplantibacillus plantarum* 256) nor the *L. plantarum* 256 supplemented in water affected the relative abundance of *Lactobacillus* in broilers' ceca compared to the control birds that did not receive any LAB. Therefore, we speculate that the initial inhibition of *C. jejuni* growth in feces observed in the silage group in Experiment 1, may be pH dependent rather than due to the presence of LAB. In contrast, a possible effect of *C. jejuni* colonization on the *Lactobacillus* abundance was observed in sequencing data, where the relative abundance of the genus *Lactobacillus* decreased after the *C. jejuni* challenge in Experiment 1, independent of treatment. With the possible impact of lower *C. jejuni* abundance with time after the challenge, the highest presence of *Lactobacillus* was observed at 20 dpi. Interestingly, a linkage between the genera *Lactobacillus* and *Campylobacter* has been previously reported by Sofka et al. (2015), where LAB were found to be significantly higher, in total cultural colony counts, in *Campylobacter-negative* samples from broiler flocks in comparison to the *Campylobacter-positive* ones. Yet, the same correlation was not observed in Experiment 2, where the relative abundance of genus *Lactobacillus* increased after the *C. jejuni* challenge, and its abundance was increasing with time after the infection, independently of treatments. Moreover, even if the genus *Lactobacillus* had at −1 dpi relatively high abundance in RR ceca in the silage and LP256 group in comparison to the control (not significant), no effect of its presence on *Campylobacter* loads was observed after the infection.

Despite the fact that all tested birds were culture negative for *Campylobacter* the day before infection (−1 dpi), we unexpectedly observed a relative abundance of 0.01% of the genus *Campylobacter* in both experiments at −1 dpi. Because the same minor presence of this genus was observed in −1 dpi samples in both experiments, although birds were sampled 1 week apart, and no *C. jejuni* were detected by culture, it is highly unlikely that the 16S amplicon sequence variants reflect the actual presence of *C. jejuni*. If *C. jejuni* would have been present in the stable before the challenge, the level of abundance detected by 16S amplicon sequencing would likely differ between Experiments 1 and 2. Furthermore, the abundance of *C. jejuni* would have been higher since it is well established that after infection, broilers rapidly accumulate high numbers of *C. jejuni* in the cecal content within 3 days (Shanker et al., 1990). Therefore, we assume that contamination in DNA extraction has occurred.

In conclusion, the current study shows that grass silage inoculated with *L. plantarum* 256 (provided as TMR) or water supplemented with *LP*256 are not effective interventions against *C. jejuni* colonization in R-308 or RR broilers. Yet, the minor reductions in *C. jejuni* observed at 1 dpi in fecal samples from R-308 suggest that this approach could still be explored and optimized for better effects. However, it should be noted that due to the cfu/g expression, the reduction in *C. jejuni* load in one group may be artificially biased compared to the other groups.

Further optimization could involve a change from a grass-based silage to a wheat-based silage, as the latter is likely to be more palatable to the birds and hence result in a larger amount of LAB consumed. This could potentially induce greater gut colonization and a stronger inhibitory effect on *C. jejuni*. However, further research is needed to confirm this hypothesis, together with the evaluation of wheat-based silage inclusion levels on the broiler's performance. It is evident from this work that *C. jejuni* presence as well as broiler type and age had much greater effects on the cecal microbiota composition than the different feed additives.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA876811, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA876811.

Ethics statement

The animal study was reviewed and approved by the Uppsala Ethics Committee for Animal Research, Uppsala, Sweden. Protocol number 5.8.18-16271/2017.

Author contributions

HWall, EI, KK, and PE obtained the funding and designed the experiments, with HWall as the project leader. EV, HWall, EI, PE, HWang, and FD obtained samples during the study. EV analyzed, interpreted, and compiled the data. LS performed the bioinformatics analysis. FD and HWang performed the plating, DNA extraction, and qPCR analysis. EV wrote the major part of the manuscript, and PE and LS some parts. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1205797/full#supplementary-material

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Campylobacter infections are the most commonly reported zoonotic disease in the EU, often linked to broiler products. Identifying strategies to reduce the occurrence of *Campylobacter* at the farm level has great potential to minimize human infections. This thesis explored the impact of *Lactiplantibacillus plantarum* 256 and algal extract dietary inclusion on broiler gut microbiota composition and inhibition of *C. jejuni* colonization. Dietary interventions proved ineffective against *Campylobacter*, however, the results contribute to a better understanding of broiler gut microbial interactions.

Eliška Eliasson received her postgraduate education at the Department of Applied Animal Science and Welfare, Swedish University of Agricultural Sciences. She received her Master's degree in Animal Nutrition at Mendel University in Brno, Czech Republic.

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