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Evaluation of early feed access and algal extract on growth performance, organ development, gut microbiota and vaccine-induced antibody responses in broiler chickens

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ABSTRACT

Hatching concepts such as on-farm hatching provide an opportunity to supply newly hatched chickens with optimal nutrition that support growth and development of a healthy gut. Brown algae contain bioactive compounds, especially laminarin and fucoidan that may improve intestinal health and immune responses. This study aimed to examine the effects of early access to feed and water posthatch and feed supplementation with algal extract rich in laminarin from *Laminaria digitata,* on growth performance, organ and microbiota development and antibody production. A total of 432 Ross 308 chicks were allotted to 36 rearing pens in a 2 × 3 factorial design with two hatching treatments and three dietary treatments. During chick placement, half of the pens were directly provided access to feed and water (**Early**) while half of the pens were deprived of feed and water for 38 h (**Late**). The chicks were fed three different starter diets until day 6; a wheat-soybean meal-based control diet, a diet with low inclusion of algal extract (0.057%) and a diet with high inclusion of algal extract (0.114%). Feed intake and BW were registered on pen basis at placement, days 1, 6, 12, 19, 26, 33 and 40. To induce antibody responses, all chicks were vaccinated against avian pneumovirus on day 10. Three chicks per pen were selected as focal animals and used for blood sampling on days 10 and 39. On days 6, 19, and 40, two birds per pen were killed and used for organ measurement and caecal digesta sampling for gut microbiota analysis using the Illumina Miseq PE 250 sequencing platform. Results showed that algal extract did not influence gut microbiota, gut development or vaccine-induced antibody responses. However, during the first 38 h, early-fed chicks consumed on average 19.6 g of feed and gained 27% in BW, while late-fed chicks lost 9.1% in BW which lowered BW and feed intake throughout the study ($P < 0.05$). Late chicks also had longer relative intestine, higher relative (g/kg BW) weight of gizzard and proventriculus but lower relative bursa weight on day 6 ($P < 0.05$). No effects of hatching treatment on microbiota or antibody response were detected. The microbiota was affected by age, where alpha diversity increased with age. In conclusion, this study showed that early access to feed but not algal extract improved the growth performance throughout the 40-day growing period, and stimulated early bursa development.

Implications

Early access to feed and water posthatch as well as early dietary interventions are suggested to improve gut health in broiler chickens. Results from this study showed that direct feed access after hatch improved growth rate, feed intake and bursa development compared to chicks deprived of feed for 38 h while supplementation of starter diets with algal extract did not affect growth performance or gut health. This implies that early feed access but not this type of algal supplementation can be used to improve growth performance in broiler chickens.

Introduction

In commercial chicken hatching systems, a delay in access to a feed of 24–72 h is common due to variation in hatching time and hatching practices (Bar Shira et al., 2005). However, researches in the last decades have shown that delayed access to feed and water for more than 36 h may result in impaired growth performance

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and increased mortality throughout the rearing period (De Jong et al., 2017). Moreover, negative effects on the development of the gastrointestinal tract (Lamot et al., 2014) and immune function (Bar Shira et al., 2005) have also been reported. Panda et al. (2015) concluded that the yolk sac is not sufficient to fully support the chicks' growth potential and development of gut and immune system and apart from providing nutrients, the presence of feed in the gut stimulates the utilisation of residual yolk sac (Bhanja et al., 2009). To deal with the setback of delayed access to feed and water in commercial hatching systems, alternative hatching concepts have been developed that provide feed and water early, such as on-farm hatching systems (De Jong et al., 2019). These new systems provide an opportunity to supply the newly hatched chicken with optimal nutrition that support growth performance and development of a healthy gut.

The concept of gut health has been widely researched and discussed in the last decade and there seems to be a direct relationship between animal performance and a healthy gut. Nevertheless, a clear definition of gut health is still lacking. Kogut et al. (2017) suggested that a meaningful definition could be "the ability of the gut to withstand infections and non-infectious stressors". They further concluded that maintained optimal gut health is dependent on three interdependent variables: the immune system, the microbiota, and the nutrition.

The gut microbiota is a highly adaptable ecosystem giving great opportunities to shape it by different interventions. Rubio (2019) stated that early life programming could be a strategy to control microbiota development in chicks and thereby improve health, welfare and productivity. Schokker et al. (2017) indicated that the early colonization of microbiota in the gut is a driver for immune development and suggested that early dietary or management interventions may be a strategy to stimulate colonization with beneficial bacteria. However, the number of studies investigating the influence of early access to feed on the development of the gut microbial composition is scarce. In turkey chicks, delayed feed access for 48 h increased the numbers of aerobic bacteria in ileal digesta day one posthatch (Potturi et al., 2005). Furthermore, De Jong et al. (2019) found less footpad dermatitis in on-farm hatched chicks compared to commercially hatched chicks, which likely was linked to a better litter quality. This indicates the need to further investigate the effect of early feed access and hatching practices on gut microbial development.

Brown algae contain the polysaccharide alginates, fucoidans and laminarin. The structure, physiochemical properties and fermentation products of these are different from the polysaccharide fractions found in most land-based plants (Dierick et al., 2009). Bioactive compounds from brown algae, especially laminarin and fucoidan, may improve intestinal health and immune response in poultry (Sweeney et al., 2017). Supplementing growing pig diets with algal extracts from *Laminaria* species containing laminarin and fucoidan has shown the potential to affect the microbial composition by reducing the *Enterobacteriaceae* counts and increasing the *Lactobacilli* spp. in the hindgut (Lynch et al., 2010). Moreover, Sweeney et al. (2017) found that supplementation with extract of laminarin or laminarin and fucoidan from *Laminaria digitata* improved growth rate, villi width and increased the gene expression of tight junction protein and cytokines in small intestine of chickens.

The aim of this study was to investigate the effects of the combination of early access to feed and water posthatch and supplementation with algal extract on chick growth performance, organ and microbiota development as well as immune responsiveness. We hypothesised that supplementing broiler starter diets with brown algal extract rich in laminarin would influence gut microbiota, facilitate gut development and improve chick's immune response. We also hypothesized that the effects would be more pronounced when feed and water were available already during hatching.

Material and methods

Incubation and hatching

A total of 500 fertile eggs, laid by 35-week-old breeders, were transported to the Swedish Livestock Research Centre, Uppsala, Sweden from a commercial hatchery, SweHatch, Väderstad, Sweden at embryonic day 18. The incubation of the eggs was performed in a conventional incubator (Petersime, Belgium) and started with a 23 h prewarming programme to reach an egg temperature of 37.78 °C. Thereafter, the egg temperature was maintained at 37.78 °C throughout the incubation period by adjusting incubator temperature and humidity. The weight loss of the eggs corresponded to 12% on embryonic day 18. At the research centre, the eggs were unloaded in the environmentally controlled poultry facility. The temperature of the eggs was checked regularly and maintained at 36–38 °C, and the temperature in the facility was regulated to keep this egg temperature. The humidity was kept at 55% ± 5%.

The chicks were hatched in the facility in a hatching pen and when the feathers of 36 chicks were dried, that hatching batch of chicks was marked with a specific colour on the feathers and each chick was randomly placed in one of the 36 rearing pens. This was then repeated 12 times until each rearing pen had 12 chicks. The chicks used in the experiment were hatched during 21 h between embryonic days 20.5 and 21.5. Since chicks had different biological ages within each pen, the chronological age (Van der Ven et al., 2009) was used and time zero was set to when the all chicks were placed in the rearing pens.

Housing

When all chicks were placed in the rearing pens, the temperature was set to 33 °C for the first three days and thereafter gradually reduced until it reached 23 °C on day 24, and was kept at this temperature throughout the study. The light was kept on for 24 h during hatch and the first two days posthatch, the dark period was then gradually increased to six h per day on day 8 until the end of the experiment on day 40. The rearing pens were raised from the floor, with the size (1.5 × 0.75 m) and equipped with wood shavings, three nipple drinkers and a feeder.

Experimental design and diets

A total of 432 Ross 308 chicks and 36 rearing pens were used in the experiment organized as a 2 × 3 factorial design with two hatching treatments and three dietary treatments, resulting in six replicates per treatment. During chick placement in the rearing pens, half of the pens were directly provided access to feed and water (**Early; E**) and half of the pens were deprived of feed and water for 38 h (**Late; L**). The chicks were fed three different dietary starter diets until day 6 and from day 7, all chicks were fed the same commercial grower diet (Lantmännen, Sweden) until day 40. The commercial grower feed was based on wheat and soybean meal (Lantmännen, Sweden) and was free from coccidiostats. The analysed chemical composition of the grower feed was (g/kg DM): ash 50, CP 212, and ether extract 68. The energy content (AME_n, g/kg DM) was calculated based on the sum of included feedstuffs energy value according to European Federation of Branches of the World's Poultry Science Association (1989) and was 14.3 MJ.

The three starter diets were a wheat-soybean meal-based control diet (**C**) a diet with low inclusion of algal extract (0.057%; **AEL**) and a diet with high inclusion of algal extract (0.114%; **AEH**; Table 1). The analysed chemical composition of the experimental starter diets is shown in Table 1. Feed samples were analysed for DM, ash, CP, ether extracts and amino acids as described by Valečková et al. (2020).

The algal extract was obtained from *Laminaria digitata* cultivated at sea on longlines in the Koster archipelago outside Tjärnö marine laboratory at the Swedish West Coast, and it was harvested and air-dried in April. Batches of 10 g of air-dried and milled algae were mixed with 200 ml 0.3 M HCl and ultra sonicated for 30 min, with an end temperature of 73 °C to precipitate alginate. The samples were directly cooled on ice and centrifuged at 1 500 rpm for 10 min. The supernatant containing the dissolved laminarin was saved and 99.8% EtOH was added to give a concentration of 90% EtOH. The samples were stored at 4 °C overnight to get a more efficient precipitation of laminarin, before they were decanted and centrifuged at 1 500 rpm for 10 min on day 2. The pellet was saved and was further washed in 90% EtOH to rinse out salts, and stored at 4 °C overnight, this was repeated on day 3. On day 4, the pellets were freeze-dried after centrifugation.

The purity of the extract was assumed to equal its β-glucan level which was 44.7% on DM-basis determined enzymatically by measuring of the β 1,3/1,6-glucan content (K-YBGL 12/16, Megazyme). In addition, the ash content was determined to be 14.95% on DM-basis and the non-starch polysaccharide content of the algal extract was determined by the Uppsala method (Theander et al., 1995), and was in total 64.5% on DM-basis of which: 51.2% was glucose, 5.2% fucose, 3.40% mannose, 3.09% galactose, 0.88% xylose and 0.69% arabinose.

Table 1
Composition of experimental starter diets to broiler chickens and calculated apparent metabolizable energy (AME_n; MJ/kg DM) and analysed chemical composition.

Item	Control	Algal extract-Low	Algal Extract-High
Ingredient composition (% as fed)			
Wheat	58.67	58.63	58.53
Soybean meal	22.49	22.49	22.49
Wheat middlings	10.00	10.00	10.00
Rapeseed oil	2.54	2.54	2.54
Limestone	2.19	2.19	2.19
Potatao protein	1.68	1.68	1.68
Monocalcium phosphate	0.71	0.71	0.71
Lysine-HCL	0.41	0.41	0.41
Methionine-DL	0.38	0.38	0.38
Sodium bicarbonate	0.39	0.39	0.39
Premix[1]	0.30	0.30	0.30
Threonine	0.16	0.16	0.16
NaCL	0.09	0.09	0.09
Algal extract	.	0.057	0.114
Analysed chemical composition (g/kg DM)			
AME_n[2]	13.4	13.4	13.4
DM	910	909	903
Ash	6.7	6.6	6.5
CP	260.4	258.5	260.2
Ether extract	51.6	52.8	52.1
Indispensable amino acids			
Arginine	14.8	15.6	13.6
Histidine	5.7	5.9	5.5
Leucine	17.9	18.6	17.3
Lysine	14.8	15.3	14.2
Methionine	6.9	7.9	7.2
Phenylalanine	11.9	12.9	11.5
Threonine	10.6	10.9	10.2
Valine	10.7	11.4	10.6
Dispensible amino *acids*			
Alanine	10.0	10.4	9.6
Aspartic acid	22.5	23.7	21.8
Cystine	4.1	4.1	4.1
Glutamic acid	52.0	54.7	50.8
Glycine	10.4	10.9	10.0
Proline	16.3	16.8	15.5
Serine	12.7	12.9	11.8
Tyrosine	8.8	9.7	8.7

Abbreviations: Control = wheat-soybean meal-based control diet; Algal extract-Low = a diet with 0.057% inclusion of algal extract; Algal extract-High = a diet with 0.114% inclusion of algal extract.
[2] Calculated based on the sum of included feedstuffs energy value according to European Federation of Branches of the World's Poultry Science Association (1989).
[1] The premixes provided per kg diet: Vitamin A: 10 000 IU; Vitamin D_3: 5 000 IU; Vitamin E: 100 mg; betain: 150 mg; Fe: 20 mg; Cu: 15 mg; Mn: 120 mg; Zink: 90 mg; Se: 0.35 mg; I: 1.3 mg.

Experimental procedure

The BW and feed intake were registered on pen basis at placement, days 1, 6, 12, 19, 26, 33 and 40. Feed conversion ratio (**FCR**) was calculated based on these values and was corrected for mortality that was registered daily. Three chicks per pen, from the middle of the hatching window, were selected as focal animals, neck tagged and used for blood sampling drawn from the jugular vein on days 10 and 39. To induce antibody responses, all chicks were vaccinated against avian pneumovirus (**APV**) on day 10, by intramuscular injection in the breast muscle with 0.5 ml of an inactivated commercial vaccine (Nobilis® TRT, MSD Animal Health). On days 6, 19 and 40, selected chickens per pen were killed, dissected and used for organ measurement and microbiota sampling. On days 6 and 19, one chick from an early hatching batch and one chick from a late hatching batch were selected and on day 40, two focal birds were used. On day six, they were stunned and killed by cervical dislocation, on days 19 and 40, they were killed by an intravenous injection of sodium pentobarbital.

Organ measurement and microbiota sample collection

On days 6, 19 and 40, the caecal content of one randomly selected caeca was collected immediately after the chicken was killed and dissected with an aseptic procedure, the sample was immediately frozen in liquid nitrogen. The caecal samples were stored at −80 °C until DNA extraction and analysis. After the caecal sample was collected, the small intestine, bursa, heart, liver, spleen, proventriculus and gizzard were dissected and measured for weight and/or length. The small intestine was defined as the proximal tip of the duodenum to the ileocecal junction. The proventriculus and gizzard with digesta were weighed together before the proventriculus was removed and the gizzard was emptied and weighed separately.

Detection of antibodies to avian pneumovirus

Sera were tested for the presence of antibodies to APV using the IDEXX APV Ab Test (#06-44300-04; IDEXX Laboratories Inc., https://www.idexx.com) indirect ELISA kit. The ELISA was performed according to the manufacturer's instructions. Samples were tested in duplicate and to increase the detection limit, sera were diluted 1:100, rather than that the recommended 1:500. Results were expressed as absorbance values at 650 nm and a cut-off value for samples deemed positive for antibodies to APV was calculated as the mean absorbance value + 2 SD for all prevaccination samples collected on day 10, n = 107.

DNA extraction, sequencing and bioinformatics analysis

The stored caecal digesta samples were thawed on ice, and 400 microliters of ASL lysis buffer (Qiagen, Germany) was added to the sample and homogenized. After homogenising 120 µl suspension

was added to a tube containing 200 mg of 0.1 mm silica beads, vortexed briefly and incubated at 95 °C for 5 min, then instantly placed on ice for 10 min. The sample was then beat-beaten on Precellys evolution homogenizer (Bertin Technologies SAS, France) at 8 000 rpm for 2 × 60 s with 30 s pauses. Followed by centrifugation of the samples at 2 500g for 1 min, and 120 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.

Two hundred sixteen ceca sample DNA extracts were alongside sequenced using the Illumina Miseq PE 250 sequencing platform at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Preceding procedures: The 16S rRNA gene V3–V4 regions were amplified using Illumina primer set (341F: CCTAYGGGRBGCASCAG, 806R: GGACTACNNGGGTATCTAAT) 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 (approximately 500 bp) 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).

The 16S rRNA gene sequencing data processing was performed as described in Sun et al. (2021), with the following modification: 1) using the truncation length of 221 bp for both forward and reverse reads; 2) the SILVA SSU Ref NR 99 138 dataset was used for taxonomic classification (Pedregosa et al., 2011); 3) the generalized UniFrac distance matrix (alpha = 0.5) was generated using the QIIME2 diversity plugin (Bolyen et al., 2019).

Statistical analysis

Growth performance and organ data were analysed for normality and homoscedasticity using the diagnostic plots of residuals in SAS (SAS Institute Inc, 2013) and were without apparent deviations. The statistical analysis was performed with the Mixed procedure in SAS (SAS Institute Inc, 2013). For the growth performance and organ measurement, the pen served as the experimental unit. The model included dietary treatment and hatching treatment as fixed factors and module as random factor, the hatching weight was included as a covariate in the model and the interaction between hatching treatment and dietary treatment was tested. For feed intake on day 2, hatching treatment was excluded from the model and the analysis was only performed on chicks from the early hatching treatment.

Organ measurements were determined repeatedly with age as an additional fixed factor and a repeated statement with Spatial Power Law (SP (POW)) matrix. Interactions between hatching treatment and age were also included in the model.

For all data, results are presented as least squares means ± SEM. Pair-wise differences in treatment least squares means were adjusted with the Tukey–Kramer method. *P*-value $\leq$ 0.05 was considered significant. For antibody levels to APV, i.e. absorbance values, data were analysed as group mean values ± 95% confidence intervals where mean values with non-overlapping confidence intervals were treated as rejecting the null hypothesis of no difference. For gut microbiota, permutational analysis of variance (**PERMANOVA**) was used to analyse whether groups of samples (i.e. age, feed and hatch treatment) were significantly different at 1 000 permutation.

Results

Growth performance

The hatching weight of early-fed chicks were 42.6 g, and the corresponding weight for late-fed chicks were 42.3 g ($P > 0.05$). During the first 38 h, the early-fed chickens consumed on average 19.6 g of feed and gained 27% in BW, while late-fed chicks lost 9.1% in BW (Table 2). This resulted in effects of hatching treatment ($P < 0.05$) on BW and feed intake throughout the study and on day 40, the early chickens weighed 5.9% more and had 5.1% higher feed intake than the late chickens. No effects ($P > 0.05$) on hatching treatment on FCR were observed throughout the study. However, on day 6, chicks fed AEL had superior FCR than chicks fed the control. An interaction between hatching treatment × dietary treatment ($P < 0.05$) was observed for FCR on days 19 and 33; however, no significant pair-wise interaction was observed when the *P*-value was Tukey-Kramer adjusted. Within the early group, an effect of dietary treatment was observed for the feed intake on day 2 where chicks fed C had higher ($P < 0.05$) feed intake than chicks fed AEL and AEH. No other effects ($P > 0.05$) of feed treatment on BW was observed.

Organ development

An effect of age ($P < 0.05$) on relative organ weight was observed for all measured organs except the bursa, where a tendency ($P = 0.07$) was observed (Table 3). The relative organ weights in g/kg BW decreased with increasing age for all organs except the spleen that was higher on day 40 than on days 6 and 19. An interaction between hatching treatment and age was observed for the relative small intestinal length and the relative weights of bursa, empty gizzard and proventriculs + gizzard, with differences between early and late chicks on day 6. On day 6, late chicks had relatively longer small intestine ($P < 0.05$), lower bursa weight ($P < 0.05$) and higher weights of empty gizzard and proventriculus + gizzard ($P < 0.05$). These observed differences could no longer be observed at d 19 or 40. No effects of dietary treatment on organ development were observed.

Antibody responses to vaccination against avian pneumovirus

All chickens were vaccinated against APV on day 10 to assess their capacity to produce antibodies to a novel antigen. The vaccination-induced antibody responses were subsequently measured in serum collected from three birds in each module, i.e. 18 birds/-group, on day 40 (Fig. 1). The results showed that overall only approx. half, 49.5 %, of the chickens tested were deemed positive for antibodies to APV and even fewer showed substantial seroconversion. The proportions of positive chickens varied slightly between the treatments groups (C-E 41.2 %; AEL-E 62.5 %; AEH-E 35.3 %; C-L 47.1%; AEL-L 52.9 %; AEH-L 60.0 %) with the AEH-E group having the lowest proportion of chickens responding to the vaccination. Nonetheless, we could not observe any significant influence of the treatments on the vaccine- induced antibody responses.

Gut microbiota analysis by illumina amplicon sequencing

After performing the quality trimming and chimera check, the 16S rRNA amplicon sequencing resulted in 10 367 647 reads in total, with a median reads per sample at 56 642. The data were then normalized by subsampling each sample according to the one with lowest sequencing reads (i.e. 7 716 reads). The alpha diversity measured by the observed amplicon sequencing variants

Table 2
Hatching weights (d0) and effects of hatching treatment, dietary treatment and the interaction between hatching treatment and dietary treatment on accumulated BW, feed intake (FI, as fed basis), and feed conversion ratio (FCR) of broiler chickens at seven different ages (day; d2-40). Least square means ± pooled SEM.

	Hatching treatment (H)			Dietary treatment (D)				P-value		
	Early	Late	SEM	Control	Algal extract- Low	Algal extract- High	SEM	H	D	D × H
BW (g)										
d0	42.6	42.3	0.186	42.7	42.4	42.3	0.227	0.214	0.382	0.274
d2	54.3	38.4	0.31	55.0	55.0	53.4	0.78	<0.001	0.219	0.301
d6	144.7	117.0	1.49	128.4	133.6	130.6	1.82	<0.001	0.141	0.864
d12	365.9	313.5	13.38	342.0	338.4	338.6	12.78	<0.001	0.963	0.823
d19	720.5	645.8	15.67	663.4	694.2	692.9	19.15	0.003	0.435	0.423
d26	1 274.4	1 178.3	22.23	1 215.8	1 218.4	1 244.8	27.50	0.005	0.715	0.498
d33	2 086.0	1 971.1	43.29	2 011.7	2 020.9	2 053.1	48.03	0.012	0.708	0.159
d40	2 919.4	2 748.6	73.78	2 800.8	2 800.9	2 900.2	81.59	0.023	0.428	0.900
FI (g)										
d2	19.6	NA[1]	2.12	21.6[a]	18.4[b]	18.8[b]	0.76	NA[1]	0.022	NA[1]
d6	97.3	70.9	1.20	86.2	83.6	82.4	1.46	<0.001	0.217	0.124
d12	368.3	310.3	9.13	331.5	342.9	343.5	10.73	<0.001	0.636	0.939
d19	863.8	765.8	17.37	787.1	825.7	831.7	21.22	<0.001	0.302	0.316
d26	1 661.9	1 508.2	25.48	1 555.7	1 601.7	1 597.6	31.13	<0.001	0.529	0.631
d33	2 712.7	2 522.9	33.20	2 584.4	2 634.5	2 633.6	40.56	<0.001	0.633	0.887
d40	4 030.7	3 825.5	58.29	3 894.0	3 948.7	3 941.6	67.50	0.006	0.785	0.930
FCR										
d2	1.68	NA[1]	NA[1]	1.82	1.51	1.72	0.111	NA[1]	0.143	NA[1]
d6	0.96	0.95	0.016	1.00[a]	0.92[b]	0.94[ab]	0.020	0.852	0.016	0.199
d12	1.16	1.18	0.031	1.13	1.18	1.20	0.037	0.572	0.348	0.592
d19	1.28	1.28	0.023	1.28	1.27	1.28	0.028	0.898	0.978	0.010*
d26	1.35	1.33	0.012	1.33	1.37	1.33	0.015	0.232	0.137	0.051
d33	1.33	1.31	0.020	1.31	1.33	1.31	0.022	0.389	0.531	0.042*
d40	1.42	1.44	0.022	1.44	1.45	1.40	0.024	0.536	0.174	0.494

Abbreviations: Early = Direct access to feed posthatch; Late = deprived of feed and water for 38 h posthatch; Control = wheat-soybean meal-based control diet; Algal extract-Low = a diet with 0.057% inclusion of algal extract; Algal extract-High = a diet with 0.114% inclusion of algal extract.
*no significant pair-wise interaction when P-value was Tukey-adjusted.
[ab]Values with different superscripts indicate difference $P < 0.05$ due to dietary treatment.
[1] Data not available.

Table 3
Effects of hatching treatment (H), dietary treatment (D), age and interaction between hatching treatment and age on relative intestinal length (cm/kg BW) and relative organ weights (g/kg BW) of broiler chickens on days 6, 19 and 40 of age. Least square means and pooled SEM.

	Hatching treatment (H)										
	Early			Late				P-value			
Items	6	19	40	6	19	40	SEM	H	D	Age	Age × H
BW, (g)	143.0[A]	742.9[B]	2 832.6[C]	117.2[A]	662.5[B]	2 674.6[C]	32.89	0.006	0.559	<0.001	0.130
Intestinal length	639.0[Ab]	188.9[Bc]	64.5[Cd]	720.6[Aa]	204.5[Bc]	68.1[Cd]	10.50	<0.001	0.382	<0.001	<0.001
Intestinal weight	95.2[A]	62.5[B]	39.3[C]	92.6[A]	64.3[B]	40.2[C]	2.38	0.996	0.499	<0.001	0.587
Bursa	2.3[a]	2.1[ab]	1.6[ab]	1.6[b]	2.0[ab]	1.7[ab]	0.18	0.109	0.387	0.070	0.047
Heart	8.8[A]	7.0[B]	6.2[C]	9.5[A]	7.2[B]	6.2[C]	0.18	0.040	0.337	<0.001	0.112
Liver	44.9[A]	34.4[B]	29.8[C]	45.1[A]	34.0[B]	29.9[C]	0.98	0.843	0.1776	<0.001	0.844
Spleen	0.69[A]	0.81[A]	0.98[B]	0.75[A]	0.85[A]	1.10[B]	0.05	0.073	0.118	<0.001	0.668
Gizzard- empty	32.4[Ab]	21.7[Bc]	9.4[Cd]	38.2[Aa]	24.7[Bc]	10.5[Cd]	0.84	<0.001	0.833	<0.001	0.013
Proventriculus + Gizzard	55.2[Ab]	37.7[Bc]	16.9[Cd]	68.1[Aa]	41.7[Bc]	18.5[Cd]	1.39	<0.001	0.980	<0.001	<0.001

Abbreviations: Early = Direct access to feed posthatch; Late = deprived of feed and water for 38 h posthatch.
[ABC] Values with different superscripts of capitals indicate difference $P < 0.05$ due to age.
[a-d] Values with different superscripts of letters indicate difference $P < 0.05$ in age × hatching treatment interaction.

(**ASVs**) had the lowest value on day 6 followed by day 19, and the highest number of ASV was observed on day 40 (Fig. 2). The observed ASVs were not associated with the dietary treatment or hatch treatment. The principal coordinate analysis plot constructed from the generalized UniFrac matrix indicated the gut microbiota were distinguished between different ages (Fig. 3). The top 25 genera (Table 4) composed 85.4% of the total sequencing reads pool. Within each hatch and dietary treatment at different ages, the average of the top 25 genera consisted of at least 81.4% of relative abundance (**RA**) (Fig. 4). The top three genera from day 6 had RA higher than 8% and *Escherichia-Shigella* had highest RA (19.3 ± 9.1%) on day 6, but significant less on day 19 (1.4 ± 1.4%) and day 40 (0.5 ± 0.7%). Ruminococcus torques group had similar RA on day 6 (15.7 ± 8%) and day 19 (14 ± 4.3%); and both were higher than day 40 (8.2 ± 2.4%). *Lactobacillus* had higher RA (8 ± 9.2%) on day 6 compared to day 19 (5.6 ± 5.1%) and day 40 (5.3 ± 5.2%), which were at similar level. Clostridia_UCG-014 had very low RA on day 6 (0.9 ± 1.8%), but RA increased rapidly already on day 19 (11 ± 6.7%) and slightly increased further to 12.9 ± 4.6% on day 40. *Faecalibacterium* also had low RA of 0.8 ± 1.7% on day 6, with a slight increase to 1.8 ± 3.3% on day 19 and a significant increase to 20.5 ± 8.2% on day 40. No impact of hatch or dietary treatment on the gut microbiota can be observed.

Discussion

In contrast to our hypothesis, supplementing broiler starter diets with brown algal extract rich in laminarin did neither influence gut microbiota nor facilitated gut development nor influence

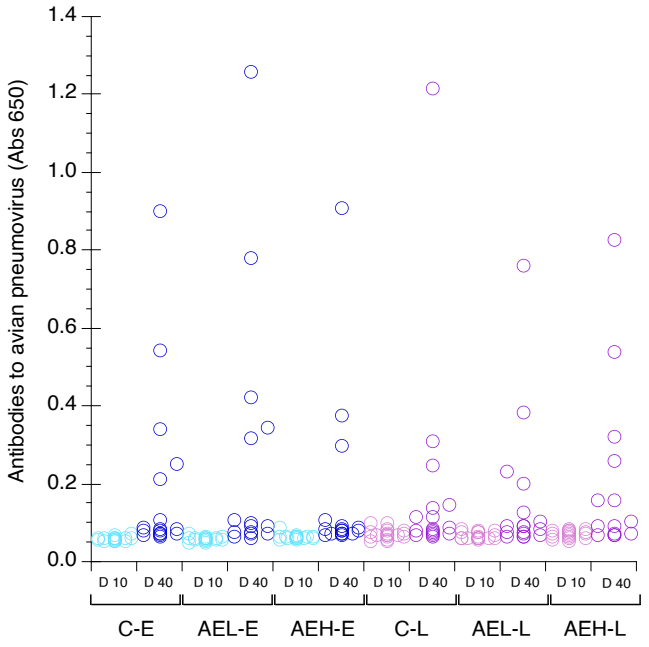

Fig. 1. Antibodies to avian pneumovirus (APV) in serum samples were collected before vaccination on day 10 (D 10) and 4 weeks after vaccination against APV on day 40 (D 40). Results are shown as absorbance 650 nm values for individual chickens in the indicated treatment groups. The cut-off value for samples deemed positive for antibodies to APV was calculated to 0.087, for details, see Material and methods. The treatment groups shown are the following (hatching × dietary treatment): Abbreviations: C-E = Control feed and early access; AEL-E = Feed supplemented with low inclusion of algal extract and early access; AEH-E = Feed supplemented with high level of algal extract and early access; C-L = Control feed and late access; AEL-L = Feed supplemented with low inclusion of algal extract and late access; AEH-L = Feed supplemented with high level of algal extract and late access.

vaccine-induced antibody responses. However, the current study showed that 38 h feed deprivation lowered the growth rate and feed intake throughout the study. It has been shown that the time from hatch to start of feed is a critical period for chick development and the following performance of the chick (Panda et al., 2015) and our results on reduced growth rate and feed intake are in agreement with several studies on feed deprivation of chicks. For example, the meta-analysis performed by de Jong et al. (2017) showed 5.5% lower BW and 4.9% lower feed intake on day 42 when chicks were feed deprived between 36 and 60 h. While in analogy with the current study, the study by de Jong et al. (2017) did not observe any effects on FCR if the feed deprivation was less than 60 h. Uni et al. (1998) did not report results on FCR; however, they showed that chicks feed deprived for 36 h had decreased villus size and crypt depth as well as unusual crypt structure. The authors therefore suggested that growth depression in feed-deprived birds may be due to impaired mucosal development. Furthermore, Pinchasov and Noy (1993) found that feed deprivation for 48 h but not for 24 h induced changes in body composition with reduction in body fat content, indicating severe negative energy balance. In addition, Pophal et al. (2003) showed that feed deprivation negatively affected both growth rate and muscle cell development. In summary, this indicates that feed deprivation inducesthe negative impact on growth rate and several physiological parameters before effects on FCR are detectable.

In contrast to hatching treatment, diet did not affect BW throughout the study, however, a negative effect on feed intake of algal extract supplementation the first 2 days posthatch was observed. This was not expected and did not remain throughout the experiment. Stimulating early feed intake is important for chick development and contrary to our results, Sweeney et al. (2017) found increased feed intake and BW in broiler chicks when extracts from *Laminaria digitata* were fed until day 13. In that study, the diets either contained a pure extract with 250 ppm lam-

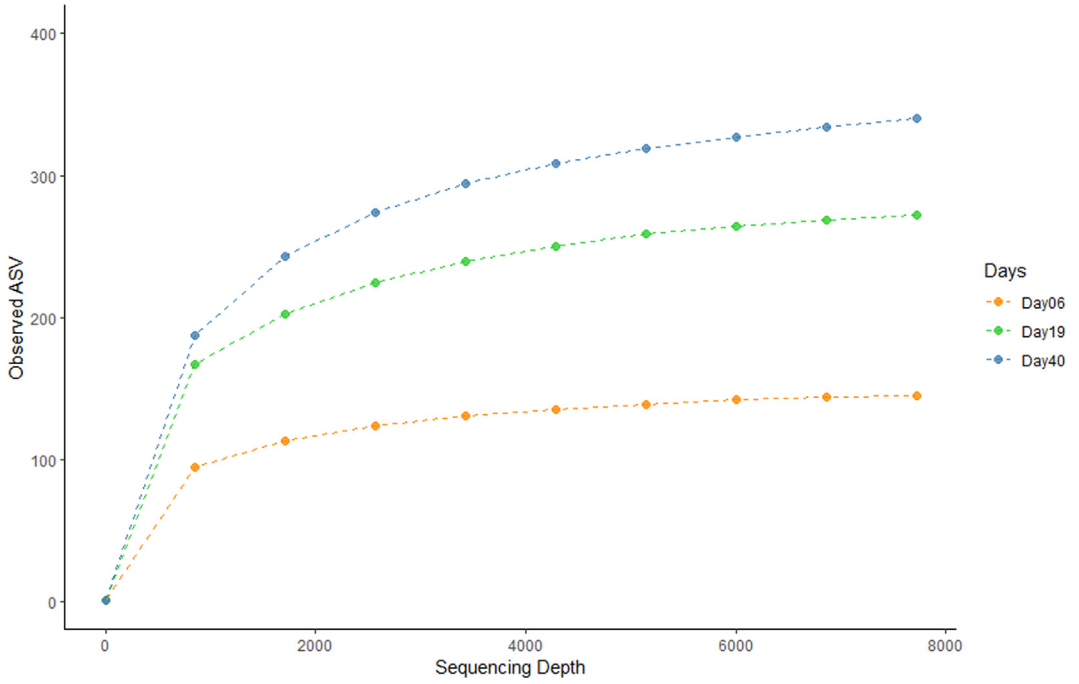

Fig. 2. The rarefaction curve of observed Amplicon Sequence Variants (ASVs) in chicken gut microbiota between different sampling days, 6, 19 and 40.

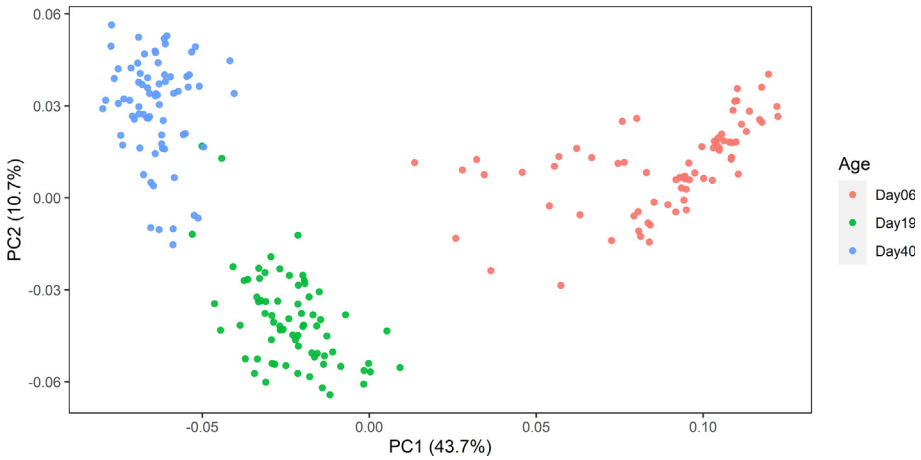

Fig. 3. Chicken gut microbiota changes over the first 40 days of life. Principal coordinate analysis of generalized unifrac distance of all gut microbiota samples sampled on days 6, 19 and 40.

Table 4
The Mean and SD of relative abundance (%) of top 25 genera of gut microbiota of broiler chickens at each sampling day.

Genus	Day06 (%)	Day19 (%)	Day40 (%)
Ruminococcus_torques_group	15.7 ± 8	14 ± 4.3	8.2 ± 2.4
Lachnospiraceae_unclassified	7 ± 3.7	9.8 ± 3	8.9 ± 2.3
Clostridia_UCG-014	0.9 ± 1.8	11 ± 6.7	12.9 ± 4.6
Faecalibacterium	0.8 ± 1.7	1.8 ± 3.3	20.5 ± 8.2
Escherichia-Shigella	19.3 ± 9.1	1.4 ± 1.4	0.5 ± 0.7
Lactobacillus	8 ± 9.2	5.6 ± 5.1	5.3 ± 5.2
Eisenbergiella	4.7 ± 7.1	6.6 ± 3.2	2.7 ± 0.9
Lachnospiraceae_NK4A136_group	2.5 ± 5.8	4.1 ± 2.7	3.4 ± 2.7
Blautia	3.1 ± 5.8	2.9 ± 1.4	1.9 ± 1
Clostridia_vadinBB60_group	0.2 ± 0.3	4.6 ± 3.4	3 ± 2.3
Colidextribacter	1.5 ± 1.4	2.5 ± 1	2 ± 0.6
[Eubacterium] _coprostanoligenes_group	0.3 ± 0.4	2 ± 1.1	3.6 ± 1.7
Clostridium_sensu_stricto_1	4.2 ± 4.9	0.8 ± 0.6	0.3 ± 0.4
Ruminococcaceae_Incertae_Sedis	1.3 ± 1.9	2.7 ± 1.6	1.2 ± 0.7
Lachnoclostridium	3 ± 1.9	1.4 ± 0.6	0.6 ± 0.2
Flavonifractor	2.9 ± 1.8	0.9 ± 0.6	0.9 ± 0.4
Klebsiella	4.4 ± 6.4	0 ± 0	0 ± 0
Enterococcus	3.6 ± 3.6	0.5 ± 0.7	0.1 ± 0.1
Oscillospiraceae_uncultured	0.2 ± 0.3	1.8 ± 0.8	2 ± 0.6
Ruminococcaceae_uncultured	0.4 ± 0.5	1.7 ± 0.5	1.6 ± 0.3
Oscillibacter	0.2 ± 0.5	2.5 ± 1.3	1.1 ± 0.3
Lachnospiraceae_GCA-900066575	0.2 ± 0.6	2 ± 1.3	1.2 ± 0.6
Negativibacillus	0.3 ± 0.6	1.6 ± 0.7	1.3 ± 0.5
Bacilli_RF39	0.2 ± 0.7	1.6 ± 1.6	1.2 ± 0.9
Butyricicoccus	0.9 ± 1	1.4 ± 0.5	0.8 ± 0.5

inarin or an extract containing 250 ppm laminarin and other components such as fucoidan, alginate and mannitol. Both extracts improved growth rate; however, chickens fed the cruder extract that also contained other fractions had a poorer FCR. The negative impact was therefore linked to the alginate and mannitol components, which have been suggested to be anti-nutritive (Sweeney et al., 2016). Although the mannitol and alginate were not determined in the extract used in the current study, a positive effect on FCR was observed on day 6 with low inclusion of algal extract, and the levels of mannitol and alginate are therefore not expected to be an issue. The inclusion levels of algal extract were based on previous studies with chickens (Sweeney et al., 2017) and pigs (Lynch et al., 2010). In the study with pigs, it was shown that 600 ppm laminarin induced negative impacts on nitrogen utilisation whereas 300 ppm laminarin showed potential health benefits by reducing the *Enterobacterium spp.* counts in colon. To our knowledge, no studies comparing different inclusion levels of algal extract on chicken production have yet been published. This study indicates that neither 0.06% nor 0.11% algal extract, corresponding to 250 and 500 ppm laminarin, have a negative impact on chicken growth performance.

Supplementation with algal extract did not affect the organ development whereas hatching treatment did. In accordance with Lamot et al. (2014), late access to feed resulted in longer relative small intestine early in life. However, no effects on relative intestinal weight was observed in current study. In the study by Lamot et al. (2014), higher relative weight on jejunum but not on ileum was observed in direct-fed chicks. In addition, Geyra et al. (2001) found that fasting for 48 h impaired small intestinal enterocyte proliferation and migration, as well as crypt and villus development. The negative effects were more pronounced in jejunum and duodenum than in ileum suggesting that the upper part of the small intestine is more sensitive to feed deprivation than the lower part. In the current study, the small intestine was measured in one section and negative effects on the upper part of small intestine might therefore not have been detected. Noy et al. (2001) showed that at 2 days of age, chicks with direct access to feed had improved intestinal development with higher relative weight of small intestine compared to chicks feed deprived for 48 h. However, the following period, during days 2–4, the feed-deprived chicks showed a higher growth rate of small intestine than direct-fed chicks, resulting in higher relative small intestine weight on day 4. This indicates that the sampling day is crucial for detecting effects on intestinal development. Differences in sampling days between studies could be a reason why no general conclusion about the effect of feed deprivation on organ development could be drawn in the meta-analysis by de Jong et al. (2017). In most studies included in the meta-analysis, no effect on organ development by feed deprivation was found. Our results showed a higher relative weight of empty gizzard and proventriculus + gizzard on day 6 in the late-fed chicks. Noy and Sklan (1997) reported that the small intestine, proventriculus and gizzard increased more rapidly in weight in relation to BW the first days' posthatch compared to other organs and the late-fed chicks had a lower BW throughout the study and on day 6, the late-fed chicks used for organ sampling weighed 26 g less than the early-fed chicks which can explain the higher relative weights in the late chicks.

Generally, the relative organ weights decreased with increasing age, which is in agreement with Valečková et al. (2020). In that study, it was also observed that for most studied organs, slow-growing Rowan Ranger chickens had higher relative weights at the same age as Ross 308 chickens and the differences in relative

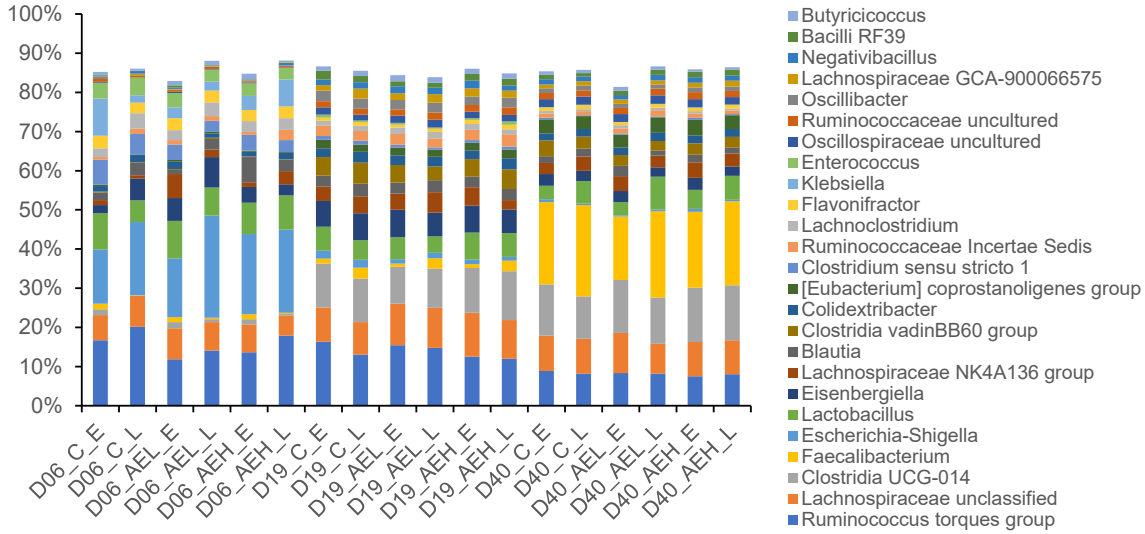

Fig. 4. The relative abundance (%) of top 25 genera of chicken gut microbiota at each feed and hatch treatment on days (d) 6, 19 and 40. The treatment groups shown are the following (hatching × dietary treatment): Abbreviations: C_E = Control feed and early access; C_L = Control feed and late access; AEL_E = Feed supplemented with low inclusion of algal extract and early access; AEL_L = Feed supplemented with low inclusion of algal extract and late access; AEH_E = Feed supplemented with high level of algal extract and early access; AEH_L = Feed supplemented with high level of algal extract and late access.

weights were linked to the high selection for muscularity and growth in Ross 308.

In contrast to gut weights, there was no effect of age on bursa weight, whereas the spleen increased in relative weight with increasing age. In addition, a higher bursa weight was observed in early-fed chicks compared to late-fed chicks on day 6. Also, a tendency ($P = 0.073$) for higher relative weight of the spleen in early-fed chicks was observed, indicating stimulation of immune organ development with early feed access, in agreement with Panda et al. (2010). Juul-Madsen et al. (2004) also showed a comprised immunological status of chicks feed deprived for 48 h, and it has been suggested that if chicks are deprived of feed and water, the available nutrients are primarily used for gut development. Provision of feed and water early both provide nutrients for the actual organ growth as well as functioning as an early antigen stimulus enabling rapid differentiation of immune organs (Bar Shira et al., 2005). Although we observed increased bursal weights for the early-fed chicks at the start of the present experimental period, which could indicate that these birds had an earlier maturation of B-cells that hypothetically could influence antibody production, we did not observe any significant effects of hatching treatment on the vaccine-induced antibody responses. The low overall response to the vaccination obviously makes any differences in antibody production difficult to detect. We have no clear explanation for this weak response albeit it has been suggested that poor immune responsiveness may be a consequence of the strong selection for improved growth rate that has occurred in broilers (Zuidhof et al., 2014). Comparable studies of early and late access to feed after hatch have shown increased antigen-specific antibody titres for early-fed chicks in responses to vaccination with live Newcastle disease virus (Panda et al., 2010) while no effect of feeding regime was observed on responses to vaccination with an inactivated infectious bursal disease virus vaccine (Juul-Madsen et al., 2004). Hence, it is possible that other factors such as the antigen/vaccine preparation used also influence whether the influence of feeding on vaccine-induced responses can be observed.

Furthermore, we were not able to detect any effect on the microbiota either by hatching treatment or by algal extract supplementation. It is known that the immune system maturation and microbiota composition are closely connected (Rubio, 2019), and changes in immune response mediated by the microbiota could therefore not be expected. Yet, an effect of age on the microbial composition was detected which is in agreement with Oakley et al. (2014) that did not find an effect of using organic acids as an additive in feed or water on the microbial composition, however, dramatic changes in the caecal microbial composition in relation to age were observed with increased complexity with increasing age. Rubio (2019) reported that the identified bacteria in the gut of broilers can be distributed in four main phyla (Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria) and the genera dominating the caecal microbiota are *Clostridium, Ruminococcus, Eubacterium, Faecalibacterium,* and *Lactobacillus* species among a number of unknown and uncultured phylotypes. The top genera identified from the different ages in current study showed that Firmicutes were the dominating phylum throughout the study, which is in agreement with other studies on meat-type chickens (Kers et al., 2018) but differs from studies on layer-type chickens where Proteobacteria are the dominating phylum until day 7 (Ballou et al., 2016). Although Proteobacteria was not the dominating phylum on day 6, *Escherichia-Shigella* was the dominating genus at this age, and a much higher relative abundance was observed on day 6 (19.3%) compared to later in life, 1.4 and 0.5% on days 19 and 40, respectively. A shift in microbial composition with a lower abundance of Proteobacteria and the family *Enterobacteriaceae*, and an increase in Firmicutes during the first weeks of life were also shown by Awad et al. (2016). In that study, the Firmicutes were dominated by the families *Lachnospriaceae, Rumonicoccaceae, Clostridiacea* and *Lactobacillacea*e, which also are well represented in the current study. On day 19, the dominating genus was *Ruminococcus_torques_group* and on day 40, it was *Faecalibacterium. Ruminococcaceae* is known for its ability to degrade complex polysaccharides (Biddle et al., 2013) and *Faecalibacterium* is known to have anti-inflammatory properties in humans, whereas its role in chickens is less known (Oakley et al., 2014). In the study by Oakley et al. (2014), *Faecalibacterium* was the dominating group on day 21 whereas on day 42, the abundance decreased and was similar to the abundance of *Lachnospiracea inecertae sedis, Oscillibacter and Roseburia*. Kers et al. (2018) concluded that microbiota

studies have to deal with many variables related to the host such as sex, age, breed and the environment such as biosecurity level, housing, litter, feed access and climate, and other variables that still remain unknown. All these variables may affect the microbial composition and the outcome of different studies. Moreover, there is still a lack of knowledge about the function of many bacterial groups in the chicken gut due to the fact that many groups are yet unidentified (Rubio, 2019). Oakley et al. (2014) stated that understanding how the changes in taxonomic composition in relation to age relate to changes in metabolic functioning and intestinal development is crucial for developing management strategies that optimize bird health and performance. However, since a lot of knowledge about the gut microbial composition is still lacking, further research is needed that enable us to relate the microbial composition to functionality.

Conclusion

In conclusion, this study showed that early feed access improved the growth rate and feed intake throughout the 40-day growing period and stimulated the early development of bursa. However, early feed access did not affect the microbial composition or immune response. Furthermore, supplementation of starter diets with algal extract rich in laminarin did not affect growth performance, microbial composition or immune response.

Ethics approval

The experiment was approved by Uppsala Animal Experiment Ethics Board (application reference number 5.8.18-1627/2017).

Data and model availability statement

The raw sequencing data have been deposited at the National Center for Biotechnology Information database (NCBI), under accession number PRJNA765180. The data that support the study findings and models are available from the authors upon reasonable request.

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Declaration of interest

None.

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