Animal Nutrition 21 (2025) 37–48

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Revealing the physical restrictions of caecal influx in broilers through the use of solid and soluble markers

Paulien Vanderghinste [a], An Bautil [a], Michael R. Bedford [b], Gemma González-Ortiz [b], Chris Lamberigts [c], Imran Aslam [d], Maarten Roeffaers [d], Christophe M. Courtin [a, *]

[a] Laboratory of Food Chemistry and Biochemistry & Leuven Food Science and Nutrition Centre (LFoRCe), Department of Microbial and Molecular Systems (M^2S), KU Leuven, 3001, Leuven, Belgium
[b] AB Vista, Marlborough, Wiltshire, SN8 4AN, United Kingdom
[c] Laboratory of Livestock Physiology & Epigenetics, Biosystems Departement, KU Leuven, 3001, Leuven, Belgium
[d] Centre for Membrane Separations, Adsorption, Catalysis and Spectroscopy for Sustainable Solutions (cMACS), Department of Microbial and Molecular Systems (M^2S), KU Leuven, 3001, Leuven, Belgium

ARTICLE INFO

Article history:
Received 24 June 2024
Received in revised form
14 November 2024
Accepted 10 December 2024
Available online 16 January 2025

Keywords:
Poultry
Caeca
Particle size
Fibre fermentation
Cellulose bead
Fluorescent polystyrene bead

ABSTRACT

A promising strategy to support broiler health and performance in a sustainable way is the enhancement of microbial fibre fermentation in broilers. This fermentation mainly occurs in the caeca, but the actual particle size range that allows caecal influx has not yet been described. This study aimed to understand the physical limitations of caecal influx as a function of broiler age by using both solid and soluble markers. In the first trial, the caecal filter mechanism was studied by microscopically visualising the caecal entrance and measuring caecal lobe development and digesta particle size as a function of age (d 8–36) for 44 broilers (Ross 308) receiving a conventional wheat-based diet. In two consecutive trials, microcrystalline cellulose beads (100–700 μm) and a combination of fluorescent polystyrene beads (5–30 μm) and chromium-ethylenediamine tetraacetic acid (Cr-EDTA) were administered to 176 and 189 broilers, respectively, at different ages (d 8–36). Results showed that the actual caecal entrance diameter is significantly reduced due to a dense villi network acting as a filter for digesta inflow. This explains the size gap between the average digesta particle size (D50) of the ileum (451–322 μm) and caeca (5–19 μm), and the outer diameter of the caecal entrance (2000–4000 μm) on d 8 to 36. In contrast to the caecal D50, cellulose beads of 700 μm already entered the caeca at 8 d of age, even though the general caecal influx of digesta particles larger than 100 μm seemed very limited. The caecal influx of the markers further exhibited large individual variation among birds. A maximum of 13.2% (d 9) and 4.3% (d 29) of the total administered soluble marker (Cr-EDTA) was detected in the caeca, 5 h after bolus administration. Both solid and soluble markers showed a larger concentration in the caeca at a young age compared to older ages ($P < 0.01$), possibly related to the limited caecal functioning early in life. These findings highlight the importance of carefully selecting the physical properties of fibres to be added as a function of age to further improve caecal fibre fermentation in broilers.

1. Introduction

Broiler production is in need of sustainable and cost-effective solutions to maintain the high performance and health status of broilers without the use of in-feed antibiotics. A promising strategy to improve gut health and performance is enhancing dietary fibre (DF) fermentation. Although DF is mainly known for its adverse effects on broiler performance due to its undigestible and anti-nutritional characteristics, more recent studies also show the potential beneficial effects of DF on health and performance (Kheravii

* Corresponding author.
E-mail address: christophe.courtin@kuleuven.be (C.M. Courtin).
Peer review under the responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2024.12.004

et al., 2017; Pourazadi et al., 2020; Vermeulen et al., 2017). Even though these effects are still not completely understood, they seem to depend on the particle size and solubility of the DF addition. Coarse insoluble DF inclusions (>1000 μm) can act as structural components in broiler diets, which can enhance gizzard functionality and retention time in the upper part of the gastrointestinal tract (GIT). As a result, increased nutrient utilisation and performance can be observed (Donadelli et al., 2019; Kheravii et al., 2017, 2018; Lentle et al., 2006; Pourazadi et al., 2020). In addition, DF fractions can be solubilised and subsequently fermented by microbiota in the broiler's hindgut, resulting in the production of short-chain fatty acids. These short-chain fatty acids can act as an additional energy source for the broiler and can stimulate the growth of beneficial microbiota, as well as control the growth and expression of invasive genes of pathogenic bacteria. Providing more substrate suitable for beneficial fibre-fermenting bacteria by increasing the fermentable DF content in broiler diets can result in an additional decrease of pathogenic bacteria in the GIT by competitive exclusion (Singh and Kim, 2021; Vermeulen et al., 2017).

Most microbial fermentation of DF in poultry occurs in the caecal lobes, which are two blind sacs located at the junction of the ileum and colon. It is hypothesised that the digesta inflow into these lobes is restricted to very small particles, solutes and fluids, although no exact cut-off in particle size has been described (Svihus et al., 2013). The marked restriction of material that enters the caeca was illustrated by Son et al. (2020), who showed that only 18% of the total excreted dry matter and 17% of the total excreted water passed through the caeca. Several studies using digestibility and transit time markers provide an indication of the physical limitations of caecal influx and illustrate the vast difference between the influx of solid and soluble markers. Ferrando et al. (1987) observed that 500 to 2000 μm fibre particles could not enter the caeca in 8- to 10-week-old broilers, but 9% to 26% of the soluble marker chromium-ethylenediamine tetraacetic acid (Cr-EDTA) did enter the caeca in 1- to 3-week-old broilers in a separate experiment by this research group (Vergara et al., 1989b). Similarly, Garçon et al. (2023) described a caecal inflow from 30% to 35% of the soluble digesta fraction in 25-day-old broilers based on the use of the soluble marker cobalt (Co)-EDTA. De Vries et al. (2014) further observed a minimal caecal influx of CrO_3, linked to the solid phase of the diet, in contrast to the abundant presence of the soluble marker Co-EDTA in the caeca in 4-week-old broilers.

Although it has been hypothesised that only small particles and fluids can enter the caeca, it has also been shown that the addition of both coarse and fine fibre particles can alter caecal microbiota composition. The increase in beneficial fibre-fermenting species and the decrease in pathogenic species in the caeca after the inclusion of fine fibre particles (80–300 μm) has been described by Boguslawska-Tryk et al. (2015), De Maesschalck et al. (2019) and Vermeulen et al. (2017). The modification of caecal microbiota by including coarse (fibre) particles (557–3000 μm) has also been demonstrated by Pourazadi et al. (2020) and Jacobs et al. (2010). It is still unclear whether these fibre particles as such can truly enter, or that only the solubles originating from the DF fraction end up in the caeca and alter the microbiota composition.

A promising strategy to increase the beneficial effects of DF on broiler health and performance is to enhance the microbial fermentation of DF in the caeca. Due to the observed physical limitations of caecal influx and the lack of a known threshold in particle size for caecal entry, a better understanding of the mechanisms of caecal influx is required. It is hypothesised that the marked restriction of the caecal influx of digesta is mainly due to the extensive network of villi present at the caecal entrance (Svihus et al., 2013). Histological research on the caecal lobes in broilers, layers, geese and quail has shown important differences in microstructures, such as villi, between the different sections (proximal, middle and distal). Well-developed villous structures are abundantly present in the proximal part of the caecal lobes, whereas more fold-like structures can be seen in the middle part and little to no folds are present in the distal part (Chen et al., 2002; Majeed et al., 2009; Pandit et al., 2018; Svihus et al., 2013). Based on this trend of extensive villi development towards the opening of the caecal lobes, a dense network of villous structures can be expected at its entrance. This network could be partly responsible for the filtering mechanism at the caecal entrance by acting as a sieve for the ileal digesta (Clench and Mathias, 1999; Fenna and Boag, 1973; Svihus et al., 2013). Our research group observed that caecal lobe weight and the size of the opening increase with broiler age, which could indicate that the particle size threshold for caecal entry also increases with age (Bautil et al., 2021). Research on the effect of age on the mode of action of this caecal filter mechanism and its histological structure in broilers is still very limited.

As outlined above, the actual size range of particles that can enter the caeca has not yet been described. In addition, multiple studies have already shown that both fine (<300 μm) and coarse (>1000 μm) fibre additions can affect the composition and metabolic functioning of the caecal microbiota and the resulting fermentation (Boguslawska-Tryk et al., 2015; De Maesschalck et al., 2019; Jacobs et al., 2010; Pourazadi et al., 2020; Vermeulen et al., 2017). It remains, however, unclear if these fibre additions enter the caeca maintaining their initial particle size, or that they affect the caecal functioning through other mechanisms. Hence, the aim of the current study is to acquire a deeper understanding of the physical limitations of caecal influx. This can enable valuable insights to explore new strategies to further increase DF fermentation in poultry.

2. Materials and methods

2.1. Animal ethics statement

The broiler trials were approved by the Ethical Committee for the experimental use of animals of the KU Leuven under accession number P140/2020.

2.2. Broiler housing, diets and sampling

Three consecutive broiler trials were conducted to study the physical limitations of caecal entrance by means of particle size in broilers. In the first part of this research, the size of the digesta particles present in the caeca was quantified for broilers fed a conventional wheat-based diet (control trial). In the second part, solid and soluble inert markers were administered to broilers in two trials to explore the particle size that allows caecal influx as a function of age.

For all three trials, conventional heating, ventilation and lighting conditions for broiler housing were applied (Aviagen, 2018). The housing temperature was set at 34 °C on d 1, gradually decreased to 21 °C at d 27 and thereafter kept constant at this temperature. The light schedule consisted of 23 h light and 1 h darkness (23:00–00:00) from d 1 to d 7, followed by a schedule of 18 h light and 6 h darkness (23:00–05:00) until the end of the trial. All broilers were kept in floor pens with fresh wood shavings and received water and feed ad libitum. Inspection of the supply of water and feed, housing conditions, health status of the broilers and mortality was carried out daily. The same basal wheat-based diet was used for the three trials (Table S1). This diet did not contain fibre-degrading enzymes but did include phytase.

2.2.1. Control trial: Caecal development and digesta particle size as a function of age

For the first trial, 44 broilers (Ross 308) at one day old were purchased from a commercial hatchery (Belgabroed NV, Merksplas, Belgium) and raised in one floor pen. At d 8, 15 and 36, a total of 18, 12 and 6 broilers, respectively, were sacrificed by electronarcosis followed by decapitation. The chyme and digesta of the gizzard, duodenum-jejunum, ileum (the section between Meckel's diverticulum and ileocaecal intersect) and caeca were collected by gentle finger stripping of the segments and pooled per 3, 2 and 1 broilers on d 8, 15 and 36, respectively to obtain 6 replicate pools of digesta at each sampling age for further analysis. The caecal lobes and the ileocaecal intersect of each broiler were also collected. Digesta samples and caecal lobes were stored at −20 °C and the intersect at −80 °C until further analysis. The weight and length of the caecal lobes and the circumference of the caecal entrance (using a ruler) were measured during sampling.

2.2.2. Cellulose bead trial (large solid marker)

For the second trial, 176 male broilers (Ross 308) at one day old were purchased from a commercial hatchery (Belgabroed NV, Merksplas, Belgium) and randomly divided over 4 floor pens. Each pen was assigned to one dietary treatment and contained 44 broilers. The dietary treatments consisted of the basal wheat-based diet (Table S1) supplemented with 1% microcrystalline cellulose beads (Cellets, Ingredientpharm, Pratteln, Switzerland) of one of 4 different particle sizes: 100, 200, 300, and 700 μm. At d 8, 15, 25 and 36, a total of 18, 12, 6 and 6 broilers, respectively, per dietary treatment were sacrificed by electronarcosis followed by decapitation. Digesta was pooled per 3, 2, 1 and 1 broilers on d 8, 15, 25 and 36 respectively to obtain 6 replicate pools of sufficient digesta at each sampling age for further analysis. Digesta collection and measurement on the caecal lobes were executed as described for the control trial (2.2.1).

2.2.3. Fluorescent polystyrene beads (small solid marker) and Cr-EDTA (soluble marker) trial

For the third consecutive trial, a total of 105 male broilers (Ross 308) at one day old were purchased from a commercial hatchery (Belgabroed NV, Merksplas, Belgium) and randomly divided over floor pens. On d 9 and - 29, a marker bolus of 1 mL was administered through oral gavage to 70 and 35 broilers, respectively. The bolus administration started in the morning, resulting in an administration starting time 9 or 4 h after the dark period at d 9 or d 29, respectively. No fasting period was applied beforehand to ensure conventional bowel movements, caecal filling and transit time. The bolus contained the soluble marker Cr-EDTA (954 μL/mL) and three types of solid fluorescent polystyrene microbeads (Spherotech, Gentaur, Kampenhout, Belgium) with average particle sizes (D50) of 5 μm (1%, wt/vol), 15 μm (0.2%, wt/vol) and 30 μm (1%, wt/vol) with a concentration of respectively 9, 9 and 26 μL/mL in the bolus. On d 9 and - 29, respectively, 14 and 7 broilers per time point were sacrificed by electronarcosis followed by decapitation at 1, 3, 5, 10 and 24 h after the bolus administration to collect 7 replicate digesta pools per time point. At d 9, digesta was pooled per two broilers to obtain a sufficient amount for further analysis. Chyme and digesta of the gizzard, duodenum-jejunum, ileum (the section between Meckel's diverticulum and ileocaecal intersect) and caeca were collected by gentle finger stripping the segments. The digesta and the emptied parts of the GIT were stored at −20 °C until further analysis.

2.3. Measurements on intestinal and digesta samples of the control trial

2.3.1. Microscopic visualisation of the caecal entrance

The tissue of the caecal entrance was embedded according to an adjusted method of Chen et al. (2002). Sections of the caecal entrance of each sampling age (d 8, 15, 36) were fixed in a 3.0% paraformaldehyde-1.0% glutaraldehyde solution for 16 h, after which they were washed with saline solution (0.9%) and cut into 5-mm tissue slices with a scalpel to obtain minimally 2 slices per caecal entrance. The tissue slices were dehydrated in ethanol series with increasing concentration (70% to 100%), cleared with toluene (99.8%), and then embedded in paraffin. These paraffin blocks were cut into 8-μm thick sections with a Leica RM2255 microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany). Sections were subsequently stained with hematoxylin (6 min) and eosin (30 s) for histological analysis. Dimensions of the caecal opening were measured using light microscopy (Nikon Eclipse 80i microscope, Nikon Inc., New York, USA) and ImageJ.

2.3.2. Determination of diet, ileal and caecal digesta particle size distribution

The particle size distribution of the diets (starter, grower, finisher) and of the ileal and caecal digesta at each sampled age (d 8, 15, 36) was measured with a Laser Diffraction Particle Size Analyzer LS 13 320 (Beckman Coulter Inc, Indianapolis, IN, USA). This device can measure particle sizes ranging from 0.375 to 2000 μm and assumes that all particles are spherical to generate a volumetric particle size distribution. Diets and ileal digesta were sieved through a 1000-μm mesh prior to this analysis due to technical restrictions (i.e. limit in particle size range) of the device. The volumetric D50 was also calculated for each measurement.

2.4. Detection and quantification of markers in digesta samples

2.4.1. Quantification of cellulose beads (large solid marker)

Ileal and caecal digesta samples of d 8, 15, 25 and 36 of broilers fed the diets enriched with cellulose beads were microscopically screened for the presence of the beads. Prior to bead screening, digesta samples were diluted as follows: 0.2 mL of caecal digesta (50.0 mg) of each replicate pool was diluted in 2.0-mL demineralised water in triplicate. Then, 0.2 mL of these solutions was placed on a microscopy glass to screen for cellulose beads. This microscopic screening was performed in triplicate for each solution to determine the presence of 100, 200 and 300 μm beads (10 × magnitude, Nikon Eclipse 80i microscope, Nikon Inc., New York, USA). Due to the large size of the 700 μm beads, their presence was determined by visually screening the diluted digesta samples.

2.4.2. Detection of fluorescent polystyrene beads (small solid marker)

The collected digesta samples were screened for the presence of fluorescent polystyrene beads in collaboration with the Roeffaers Lab (KU Leuven, Belgium). After lyophilisation and grinding of ileal and caecal digesta samples of broilers that received the marker bolus, 50 mg digesta of each sample was suspended in 1 mL demineralised water. 250 μL of this solution was mixed with 250 μL agar and poured into a 9.4 mm × 10.7 mm × 9.3 mm well of an Ibidi μ-Slide 8 well for visualisation with a Leica TCS SP8X (Leica Microsystems GmbH, Germany). Three excitation and emission wavelength combinations were used simultaneously to detect the fluorescent polystyrene beads based on the optimal

excitation and emission wavelength ranges of each bead type (wavelengths provided in Supplementary Table S2). Tile scans of the complete wells were made by capturing images of 517 μm × 517 μm and a z-section depth of 1.2 μm (405 nm excitation) or a z-section depth of 1.7 μm (590 nm excitation) with a 10× magnification objective lens using Leica Application Suite X software (LAS X, Leica Microsystems GmbH, Germany). Image processing and bead counting was done through LAS X software and ImageJ, after which the number of beads on each image was summed to obtain a total bead count per tile scan and digesta replicate pool. Seven replicate pools of ileal and caecal digesta samples of d 8 and 25 of the control trial (2.1.1) were utilised in this analysis as control samples to validate the described bead detection method.

2.4.3. Quantification of Cr-EDTA (soluble marker)

Ileal and caecal digesta samples of broilers that received the marker bolus were lyophilised, ground, and subjected to an acid closed vessel digestion. Two reference samples of 100 mg Beech BR100 and three negative controls were included in each analysis. Eight milliters HNO_3 (69%) was added to digesta samples (100 mg) of each replicate pool of both ages (d 9 and - 29). After a predigestion of 30 min, the samples were digested in a MARS 6 microwave (CEM, Matthews, North Carolina, United States) in closed MARSXpress Teflon tubes for 70 min at 180 °C (including 25 min warm-up and 25 min cool-down time). Next, 42-mL Milli-Q water was added to each digested sample. The samples were further diluted 4-fold with Milli-Q water, after which the solubilised Cr was measured through inductively coupled plasma spectrometry (ICP-MS; Agilent 7700x, Agilent Technologies, Santa Clara, California, United States). This resulted in an ileal and caecal Cr concentration (mg Cr/kg dry digesta) per replicate, which was converted to the total Cr content in the ileum and caeca based on the total digesta dry weight collected per replicate during sampling. The percentage of Cr found in the caeca compared to the total administered Cr in the bolus was also calculated.

2.5. Statistical analysis

Statistical analyses were performed using JMP Pro 16 Software and R Statistical Software (v4.0.5; R Core Team, 2021). The normality of all datasets was evaluated through a density plot and quantile–quantile plot. The means of full caecal lobe weight, lobe length and caecal entrance diameter were calculated using sample sizes of 18, 12 and 6 broilers, respectively, at d 8, 15 and 36, due to the limited digesta sample size available from the younger birds. Broilers that showed signs of intestinal disease were excluded from this dataset. Significant differences in the mean full caecal lobe weight, lobe length and D50 of caecal digesta ($n = 6$) between age groups were identified by performing a one-way ANOVA and significantly different means were further identified using a Tukey's test. Differences between mean caecal entrance diameter and cellulose bead counts in digesta ($n = 6$) were identified using a Wilcoxon Rank Sum test due to the non-normality of the data. Significant differences between mean total fluorescent bead counts in ileal and caecal digesta of the treated broilers compared to the control broilers ($n = 7$) were identified by performing a one-way ANOVA and significantly different means were further identified using a Student's *t*-test. Significant differences in mean ileal and caecal Cr concentrations between the measured time points and in ranges of caecal influx ratio of Cr at different broiler ages ($n = 7$) were identified using the Wilcoxon Rank Sum test. Differences between means were considered significant at $P < 0.05$ and interpreted as a trend at $0.05 \leq P \leq 0.10$.

3. Results

3.1. Control trial: caecal development and digesta particle size as a function of age

3.1.1. Development of caecal lobes and entrance as a function of age

The evolution of caecal lobe weight, length and circumference of the caecal entrance measured on fresh caecal lobes are shown in Fig. 1. The average weight of both full caecal lobes increased with age from 1.58 ± 0.40 g on d 8 to 12.18 ± 5.25 g on d 36 ($P < 0.001$). This corresponded to a relative weight of 0.87 ± 0.30 g/100 g BW on d 8 and 0.46 ± 0.20 g/100 g BW on d 36. The average length of the caecal lobe increased from 7.1 ± 0.7 cm on d 8 to 17.6 ± 2 cm on d 36 ($P < 0.001$). The average diameter of the caecal entrance measured on fresh samples increased from 0.2 ± 0.04 cm on d 8 to 0.4 ± 0.05 cm on d 36 ($P < 0.001$). Figure 2 shows the stained sections of the embedded caecal entrances at d 8, 15 and 36. The visualisation of the caecal entrance showed that the true inner diameter of the entrance is markedly reduced due to additional tissue layers inside this entrance at all studied ages. These tissue layers consist of a submucosa layer with protrusions towards the inside of the caeca, on which a villi layer is attached. The average of the geometric mean diameters of the embedded caecal entrances measured 0.1133 ± 0.0224 cm, 0.1943 ± 0.0457 cm and 0.2143 ± 0.0679 cm on d 8, 15 and 36, respectively.

3.1.2. Ileal and caecal digesta particle size distribution as a function of age

The average diet, ileal and caecal particle size distribution of broilers fed a wheat-based diet as a function of age is shown in Fig. 3. There was a significant reduction in particle size as digesta moved from the ileum to the caeca at all studied ages. The D50 of the ileal digesta on d 8, 15 and 36 was 451 ± 49 μm, 322 ± 25 μm and 409 ± 24 μm, respectively, in comparison to the diameters of the caecal digesta of 5 ± 3 μm, 9 ± 2 μmand 19 ± 8 μm at the same age. The D50 of the caecal digesta on d 8 was smaller than on d 36 ($P < 0.001$). On d 8 and 15, 70% of the caecal digesta particles were smaller than 10 μm. On d 36, this upper limit for 70% of the particles increased to 30 μm. However, particles up to 400 μm were also present in caecal digesta of d 8. The geometric mean diameters of the caecal entrance (1133–2143 μm) vastly exceeded the caecal digesta D50 (5–19 μm) at every studied age.

3.2. Detection and quantification of markers

3.2.1. Detection and quantification of cellulose beads (large solid marker)

All sizes of cellulose beads were found intact in the feed, along the GIT and in the excreta, demonstrating their inertness and suitability as particle size markers in broilers (Fig. S1). Individual body weight and gut development parameters were also not significantly affected by the inclusion of the cellulose beads. Figure 4 shows the cellulose bead concentration in the caecal digesta for each screened replicate pool per age and per bead size. All sizes of cellulose beads (100–700 μm) were detected in at least one replicate pool at all studied ages (d 8–36), although the bead concentration in the caeca markedly varied between the replicate pools. A tendency towards higher bead concentrations at d 8 compared to d 36 could be observed irrespective of bead size, and this age effect was significant for the 100 μm and 300 μm beads ($P < 0.001$). As the bead count per gram of beads differed for each bead size (Table S2), the bead concentrations in digesta could not be compared directly between the different bead sizes.

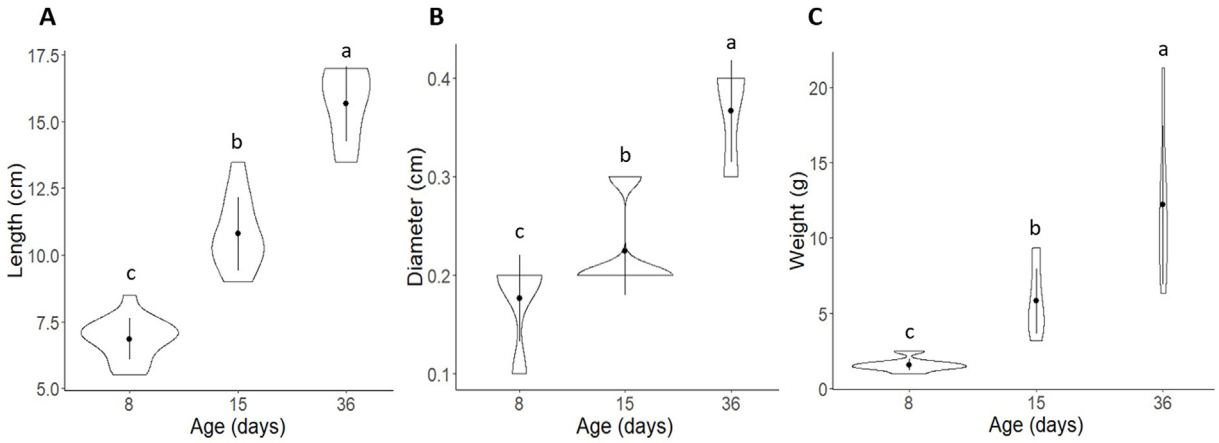

Fig. 1. Violin plots of the length (A), entrance diameter (B) and full weight (C) of fresh caecal lobes of the control trial measured during sampling at d 8 ($n = 18$), d 15 ($n = 12$) and d 36 ($n = 6$). Dots represent the mean value per age, vertical lines represent the Q1 to Q3 boxplot quantiles. [a–c] Different letters indicate a significant difference between mean values of age groups ($P < 0.05$).

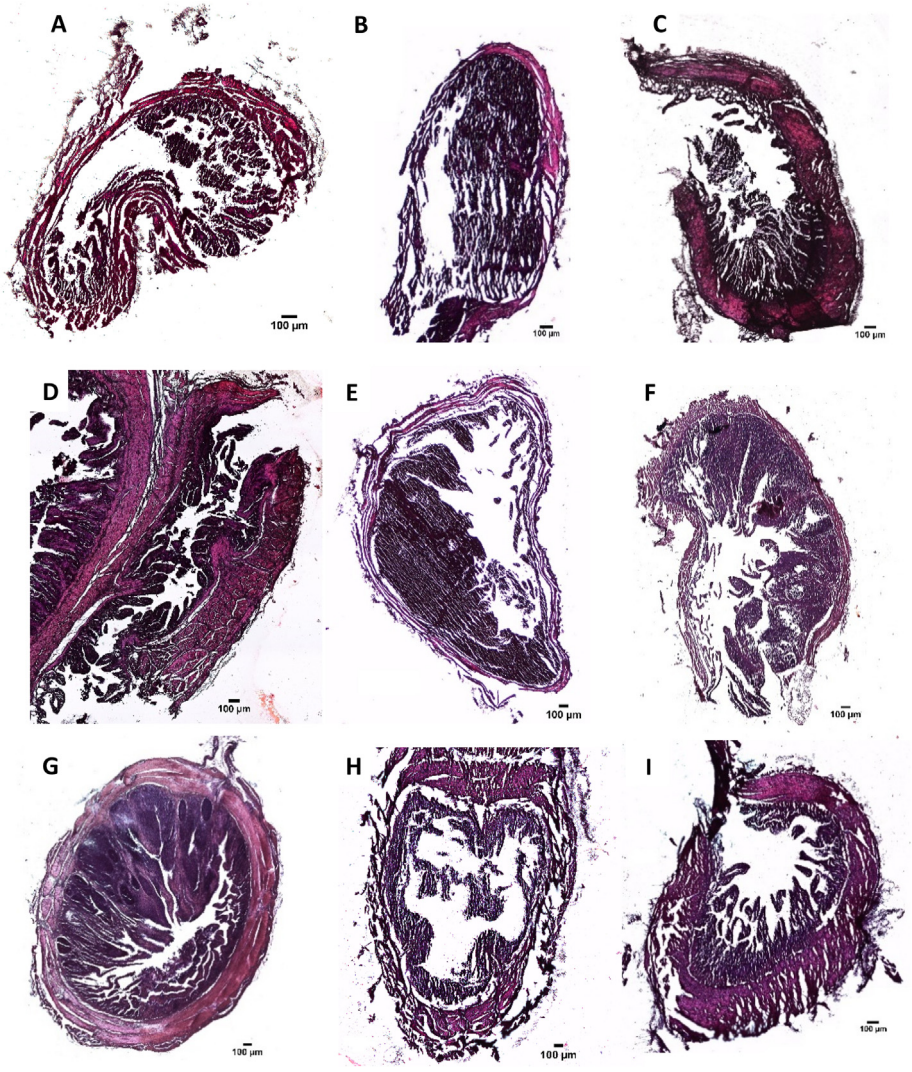

Fig. 2. Microscopic images of 8-µm thick sections of caecal entrance tissue embedded in paraffin and stained with hematoxylin and eosin, from caecal lobes of the control trial collected at d 8 (A, B, C), d 15 (D, E, F) and d 36 (G, H, I). The muscular outer layers are stained in light pink, the inner submucosa and villi network are dark purple.

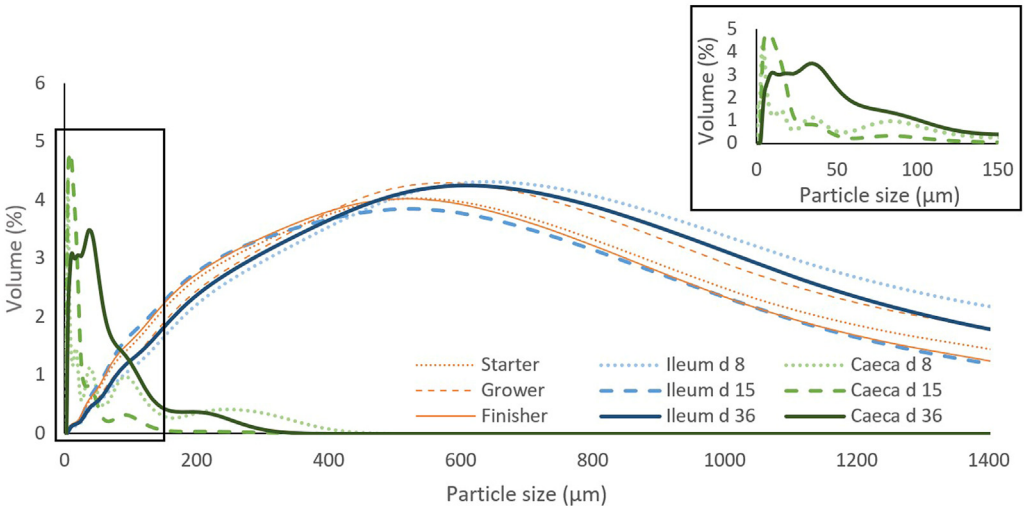

Fig. 3. Average volumetric particle size distribution of the diets, ileal and caecal digesta of the control group collected at d 8, 15 and 36 ($n = 3$ for each diet, $n = 6$ per age group for digesta), expressed as volume percentage. Orange lines represent the diets, blue lines the ileal digesta and green lines the caecal digesta. Dotted, dashed and solid lines represent the particle size distribution at an age of respectively 8, 15 and 36 d.

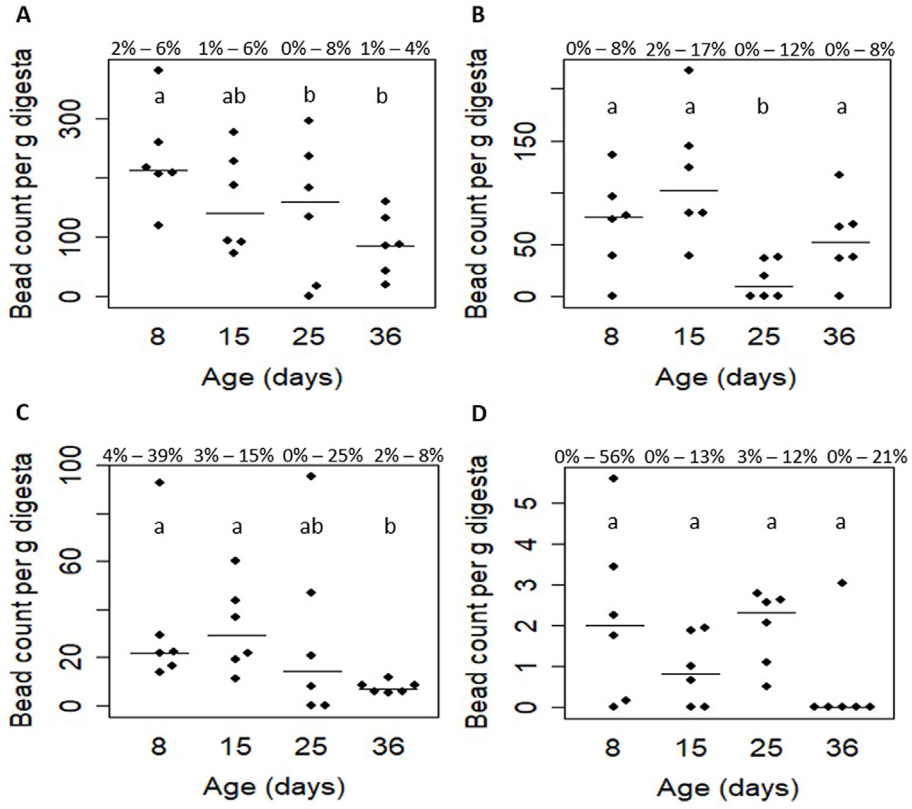

Fig. 4. Beeswarm plots showing the effect of bead size (A: 100 μm [1739 beads per g feed], B: 200 μm [1089 beads per g feed], C: 300 μm [556 beads per g feed], D: 700 μm [55 beads per g feed]) on bead concentration in caecal digesta of broilers at different ages (d 8, 15, 25 and 36, $n = 6$ per age group). Points represent the bead count per g digesta of each biological replicate and a horizontal line indicates the average bead count per g digesta per age. [a, b] Different letters indicate a significant difference in mean bead concentration between age groups ($P < 0.05$). Above each beeswarm, the ratio of bead concentration in the caeca compared to the ileum is given per bead size and age.

The ratio of the bead concentration in the caeca over that in the ileum is also shown in Fig. 4 per age group and bead size. This ratio indicates the caecal influx of the beads and ranged from 0.6% to 55.9% over all treatments and ages, showing large variability between biological replicates. The ratio was not affected by bead size or broiler age.

3.2.2. *Detection and quantification of fluorescent polystyrene beads (small solid marker)*

The total count of fluorescent polystyrene beads of 5 μm in digesta of the treated broilers 1 h after bolus administration only differed from the control broilers for the ileal digesta samples at the youngest age (d 9) ($P < 0.001$), as shown in Fig. 5. No significant differences in

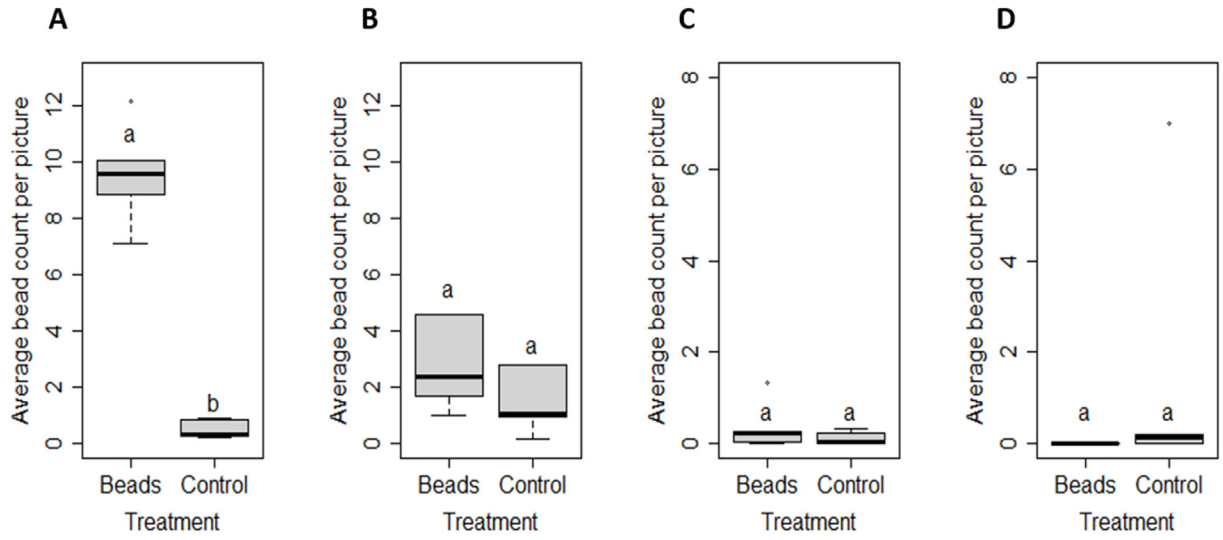

Fig. 5. Boxplots of total counts of 5-μm fluorescent polystyrene beads per tile scan by ImageJ for ileal digesta on d 9 (A), ileal digesta on d 29 (B), caecal digesta on d 9 (C) and caecal digesta on d 29 (D) of broilers 1 h after bolus administration compared to control broilers ($n = 7$ per age group). [a, b] Different letters indicate a significant difference in mean bead count between treatments ($P < 0.05$).

ileal (d 29) and caecal (d 9 and - 29) counts of 5 μm beads between the treated broilers and the control were detected. The low concentrations of the beads in the administered bolus, especially of the 15 μm and 30 μm beads due to their larger particle size, hindered their detection in all types of digesta samples. Strong autofluorescence signals of other digesta particles were observed in both the control and bead-treated ileal and caecal digesta samples, which further complicated the detection of the beads (Fig. S2 and Fig. S3).

3.2.3. Detection and quantification of Cr-EDTA (soluble marker)

Figure 6 shows the total Cr content in the ileal and caecal digesta over time at d 9 and - 29 for each replicate pool and the average per age and time point, starting 1 h after bolus administration. The first presence of Cr in the ileum and caeca was already observed 1 h after bolus administration on both d 9 and - 29. The ileal Cr content at this first time point was higher at d 9 compared to d 29 ($P = 0.022$), but this age effect disappeared at later time points in the ileum. In contrast, the caecal Cr content remained higher at d 9 compared to d 29 at time points 3 h ($P = 0.001$) and 5 h ($P = 0.004$). The maximal Cr content was observed 3 h after bolus administration in the ileum at both ages, while a first maximal caecal Cr content was observed at the 5 h time point for both ages. The Cr content in the caeca compared to the total administered Cr in the bolus ranged from 3.5% to 13.2% (d 9) and from 1.7% to 4.3% (d 29) at the 5 h time point, with higher values at a younger age ($P = 0.004$). Relatively large amounts of Cr were retained in the caeca for up to 24 h at both ages, with a large variation between biological replicate pools. The total Cr content in the ileal and caecal digesta 1 h and 3 h after bolus administration and the calculated caecal influx ratio are shown in Fig. 7. Due to the high variation in both ileal and caecal Cr content, the results are shown per replicate pool. The unexpected mortality of three broilers between the period of bolus administration and sampling resulted in 6, 5 and 6 replicate pools instead of 7 in Fig. 7 for the time points 1 h and 3 h (d 9) and 1 h (d 29), respectively. The ratio of the Cr content in the caeca compared to the ileum at the 1 h time point can indicate the caecal influx of Cr or the flow of Cr from

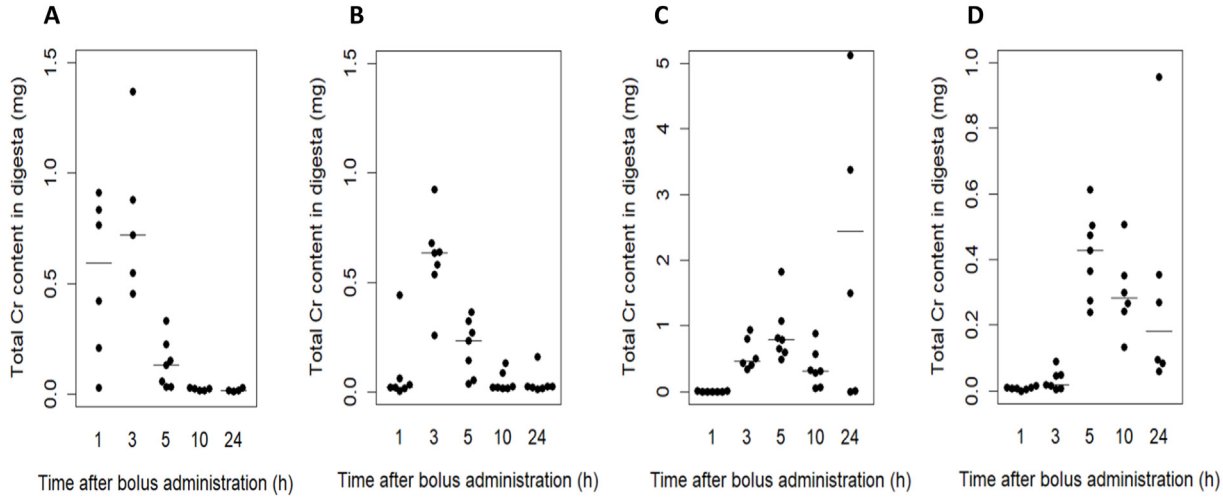

Fig. 6. Beeswarm plots of the total Cr content in ileal (A: d 9, B: d 29) and caecal (C: d 9, D: d 29) digesta collected at 1, 3, 5, 10 and 24 h after bolus administration at d 9 and 29 ($n = 7$ per time point and per age group). Points represent the total Cr content (mg) per biological replicate and a horizontal line indicates the average per time point.

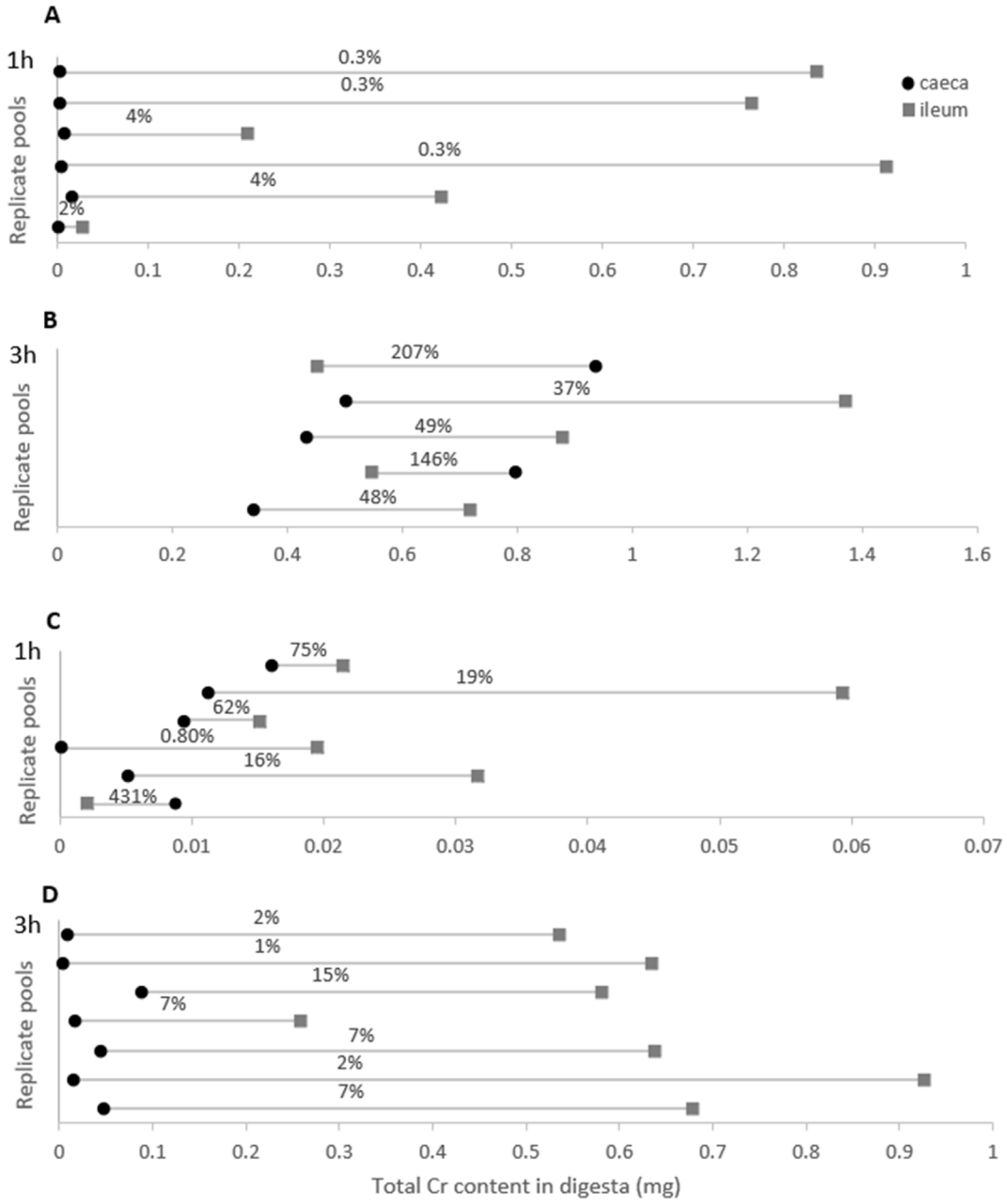

Fig. 7. Dumbbell plots of the Cr content (mg) in caecal (black dots) and ileal (grey squares) digesta collected 1 and 3 h after bolus administration at d 9 (A, B) and d 29 (C, D) of each biological replicate, corrected for digesta dry weights ($n = 5–7$ per age group). The ratio of the Cr content in the caeca compared to the ileum per biological replicate is given as percentage above each measurement.

the ileum into the caeca. This ratio varied greatly between replicate pools due to variations in both the ileal and caecal Cr content. The caecal influx ratio tended to increase with age from 0.3% to 4% on d 9 to 0.8% to 75% on d 29 ($P = 0.055$). One replicate pool on d 29 already had a higher Cr content in the caeca compared to the ileum at the 1 h time point, resulting in a caecal influx ratio above 100% (430%). At the 3 h time point, two replicate pools showed a caecal influx ratio above 100%.

4. Discussion

The evolution of the broiler's caeca and the caecal digesta particle size distribution with age were studied as a first step to reveal the physical restrictions of caecal influx. An overall increase in caecal dimensions with age was observed, such as the enlargement in caecal diameter, lobe length and full lobe weight from d 8 to d 36. This illustrates the rapid development of the caeca with age and can also indicate an increase in caecal influx of digesta and fermentation capacity with age (Svihus et al., 2013). The relative full caecal lobe weights and dimensions measured in this study correspond to previous findings in literature when broilers were offered a wheat-based diet (Bautil et al., 2021). The observed increase in caecal weights and dimensions could also be expected based on other studies that recorded caecal development with age in broilers and other young poultry species (AbuAli et al., 2019; Martínez et al., 2021). The sections of the embedded caecal entrances displayed a similar increase in diameter with age, but more importantly, showed that the actual inner diameter of the caecal entrance,

relevant for digesta influx, is markedly reduced due to a network of submucosa protrusions and villi. Additional mucus layers can also be expected to be part of this network but were probably lost during the embedding procedure. The visualisation of this network supports the hypothesis that the numerous villi at the caecal entrance act as a filter for incoming digesta particles, as described by Clench and Mathias (1999), Fenna and Boag (1973) and Svihus et al. (2013). This filtering effect was further confirmed by the large shift in D50 from the ileal digesta (409–451 μm) to the caecal digesta (5–19 μm). The lack of difference between the diet and ileal digesta particle size is mainly due to the required pre-sieving step before laser diffraction analysis. The presence of the villous network at the caecal entrance also explains the large size gap between the measured outer diameter of the caecal entrance (0.2–0.4 cm) and the actual D50 of the digesta inside the caeca at all studied ages. The villous network already seemed to be developed in the first week after hatch, but its firmness could however greatly vary with age. It appeared to be more brittle and was harder to visualise in detail at d 8 compared to d 15 and - 36, which can indicate a decreased filtering capacity of the villous network at a younger age. However, the smaller size of the caecal entrance tissue on d 8 compared to d 15 and - 36 could also have increased the difficulty of the visualisation procedure in general. Nonetheless, it is clear that this villous network at the caecal entrance restricts digesta inflow at all studied ages.

Based on the increase in the caecal physiological parameters and the caecal digesta D50 with age, the size of digesta particles that can pass through the caecal filter can also be expected to increase with age. The actual particle size range that allows caecal influx as a function of age was assessed by means of solid and soluble markers in two consecutive broiler trials. The microcrystalline cellulose beads that were used as the large solid marker in this study seemed to be a suitable marker in the broiler's GIT due to their inertness along the GIT and the absence of an effect on individual body weights and gut development parameters. As previously confirmed in other studies, the soluble marker Cr-EDTA also seemed to be a suitable marker due to its inertness and accurate detection in the digesta (Iji, 2007; Vergara et al., 1989a, 1989b). The first appearance of Cr in the ileum and the caeca in this study was already detected within 1 h after bolus administration. This fast transit of liquid markers to the small intestine corresponds to the report regarding early marker appearance in broilers and quail by Fenna and Boag (1973) and Vergara et al. (1989a, 1989b). The direct administration of the bolus into the crop through the oral gavage technique used in the current study may have additionally accelerated the passage of the markers to the other GIT parts compared to marker intake through regular feeding. The major fraction of Cr was found after 3 and 5 h respectively in the ileum and caeca, corresponding to the expected digesta transit times in these GIT parts (Svihus and Itani, 2019). However, at these later time points, which correspond to the expected time for digesta to reach the caeca, the caecal Cr content already exceeded the ileal Cr content in multiple broilers at d 9, indicating an accumulation of Cr in the caeca and an evacuation of ileal content. The frequency of caecal emptying in broilers is estimated to be every 2 to 8 h based on previous own research (data not shown). Based on these findings, the data from the 1 h time point seemed the most reliable in estimating the flow of markers from the ileum to the caeca, with a minimal effect of caecal accumulation or retention of the markers at d 9. The same time point was used at d 29 to allow a correct comparison between both ages. The caecal influx ratios at the 3 h time point are also shown in Fig. 7, but may already be affected by caecal emptying and accumulation that are still poorly understood. To study caecal marker retention, all time points were taken into account (Fig. 6). Following this reasoning, the detection of the fluorescent polystyrene beads was also performed on the digesta collected 1 h after bolus administration for the estimation of their caecal influx. These fluorescent polystyrene beads were used as small solid markers but seemed less suitable for this study, as only the 5 μm beads were detected in the ileal digesta on d 9 and no beads were detected in the caecal digesta. This mainly seems due to the low administered concentration of the beads (9%–26% [vol/vol] of bead solutions in the bolus), regarding the limited amount of caecal influx of small particles and fluids measured in this paper and previous studies (Garçon et al., 2023; Vergara et al., 1989a,b). In addition, the beads were difficult to detect in the digesta matrix due to their similar size to digesta particles and the interference of the autofluorescence of feed and digesta compared to the relatively low administered bead concentrations. Using higher bead concentrations in the oral bolus and fluorescent coatings that do not overlap with the autofluorescent signal of feed and digesta could improve their suitability as a marker for studies on the broiler's caeca. Preliminary screening of the samples obtained at later time points than 1 h did not show a better detection of fluorescent beads and were hence not analysed for this marker type.

An overall low caecal influx ratio was observed for both the solid and soluble markers, although this ratio varied between replicate pools. The average caecal influx ratio of the cellulose beads was only 5%, with no significant effect of age or bead size. Despite this low influx, the presence of cellulose beads with particle sizes up to 700 μm in the broiler's caeca is remarkable, given that only 1% to 15% of the caecal digesta particles of the unsupplemented broilers were larger than 100 μm, and no particles larger than 500 μm were detected. The cellulose beads, however, differ from other digesta particles and from the native cellulose already present in the feed due to their dense microcrystalline structure, spherical shape and smooth surface. This might have facilitated their caecal entry, for example, by pushing through the villous network instead of being withheld as expected for light, non-spherical digesta particles such as wheat bran. The wide range of the caecal influx ratio of the cellulose beads (2% to 56%) over the different replicate pools shows that a high caecal marker concentration of these beads could also be achieved. A similar broad caecal influx range with a high maximum was observed for the soluble marker (0.3% to 75%) 1 h after bolus administration. It is important to note that the reported influx ratios of the cellulose beads, administered through continuous feeding, can be an overestimation if the potential accumulation of markers in the caeca over time is considered (Svihus et al., 2013; Vergara et al., 1989a). On the other hand, the caecal influx ratios of the soluble marker can be an underestimation as these were calculated based on marker contents measured 1 h after marker administration. Although using this 1 h data minimizes the effect of caecal emptying and accumulation, later time points are considered more biologically relevant regarding caecal transit time (Svihus and Itani, 2019). These later time points were taken into account when studying caecal retention of the markers, but were not suitable for the calculation of the caecal influx ratio as outlined above. Aside from the infrequent caecal emptying and the possible accumulation of material in the caeca, the inflow of material into the caeca is assumed to be non-continuous and can additionally complicate the measurement and interpretation of caecal influx. A vastly higher caecal influx ratio of the soluble marker compared to the solid marker could have been expected based on the reports by De Vries et al. (2014), Svihus et al. (2013) and Vergara et al. (1989a, 1989b), but was not observed in this study. The absence of this difference may be caused by a greater accumulation of the cellulose beads in the caeca due to their larger size which may facilitate their retention in the caeca once they have entered, and their administration through continuous feeding instead of through a single bolus as used for the soluble marker. Aside from the caecal influx

ratio, the maximal Cr content found in the caeca compared to the total amount of administered Cr can indicate the extent to which the soluble digesta fraction can enter the caeca, which ranged from 1.7% to 13.2% in this study, 5 h after bolus administration. Although these values align with the few other studies that quantified caecal influx in broilers, the possible accumulation and infrequent filling and emptying of the caeca should be taken into account for a correct interpretation. It is described that caecal digesta dry matter accounts for 18% of the total excreted dry matter in broilers and that 9% to 35% of the orally administered soluble markers Co-EDTA and Cr-EDTA was found in the caeca, depending on broiler age (Garçon et al., 2023; Son et al., 2020; Vergara et al., 1989b). So, despite the non-continuous caecal inflow and outflow dynamics complicating the interpretation of the obtained marker data, this paper confirms that even the caecal influx of the liquid fraction seems very limited. Together with the results on caecal digesta particle size distribution, the current results suggest that a particle size of less than 100 μm is required to achieve maximal caecal access. Although larger particles have also been demonstrated to affect caecal fermentation and microbiota (Boguslawska-Tryk et al., 2015; De Maesschalck et al., 2019; Pourazadi et al., 2020; Vermeulen et al., 2017), these results indicate that they will only enter the caeca in very limited quantities. Hence, despite the use of different marker types and time points in this study, the exact quantification of the fraction of ileal digesta particles that can actually pass the caecal filter to enter the caeca is still hindered by the complex caecal filling and emptying mechanisms and the important role of broiler age. Aside from particle size, other physicochemical characteristics such as solubility, viscosity and molecular weight are also expected to affect the caecal entrance of digesta (Svihus et al., 2013). However, this study did highlight the importance of particle size for caecal influx and provides an indication of the particle size range allowing this caecal influx, within the studied size ranges of the used solid (100–700 μm) and soluble markers. These new insights can be of use to further improve the maximal fibre fermentation capacity in the caeca.

In addition to particle size, broiler age also seemed to be an important factor when studying the physical aspects of digesta influx into the caeca. The caecal concentration of 100-μm and 300-μm beads, the total caecal Cr content at time points 3 and 5 h and the maximal percentage of Cr in the caeca compared to the total administered Cr were all higher at a younger age (d 8–9) compared to an older age (d 29–36). This contradicts the observed increase in caecal dimensions and caecal digesta D50 with age, as well as the increase in the caecal influx of Cr with age as observed by Vergara et al. (1989b). The ileal and caecal Cr contents as a function of time (Fig. 6) can indicate a two-sided effect of age on the transit time and retention time of this marker in the GIT. Firstly, the higher ileal Cr content at d 9 compared to d 29 1 h after bolus administration may imply that the precaecal transit time of this marker is shorter at d 9 compared to d 29. Secondly, the increased caecal Cr concentrations at a younger age can be due to the faster passage of liquid digesta material at a younger age as confirmed for the ileal data above, which was also seen by Vergara et al. (1989a) for the total tract transit time of Cr-EDTA. However, these authors assigned this age effect to the increase of caecal influx with age, while the current study shows that the passage is already faster in the GIT parts prior to the caeca. Similar to the liquid Cr marker, the results of the cellulose beads show a tendency towards a higher caecal marker concentration at a young age compared to an older age. These higher caecal marker concentrations at a younger age compared to an older age could also be the result of a greater caecal retention of the marker in younger birds. Other studies have shown that digesta can remain in the caeca for longer than 12 or 24 h, leading to increased retention times of certain markers (Garçon et al., 2023; Hinton et al., 2000; Vergara et al., 1989a; Warriss et al., 2004). This aligns with the high caecal Cr concentrations observed 24 h after bolus administration. In addition, a longer transit time of the cellulose beads in the GIT at a young age compared to an older age was observed in an extra transit time experiment (data not shown), which may also be attributed to greater caecal retention of this relatively large solid marker. This means that the increased marker concentrations at a young age compared to an older age can reflect their accumulation in the caeca and may indicate a more restricted caecal outflow early in life rather than a more restricted inflow at an older age. This more restricted outflow may result from less contractile activity of the caeca lobes in young birds, which can be related to the previously reported limited functioning of the caeca at a very young age. For example, Svihus et al. (2013) expect that the full fermentation capacity of the caeca is not reached before d 28, which is supported by observations of increased levels of fermentation products formed in the caeca with increasing broiler age (Fischer, 2003; Lee et al., 2017). The increase in fibre fermentation capacity with broiler age is also reported by Bautil et al. (2019), where increased solubilisation and fermentation of arabinoxylan was measured in broilers at 21 d compared to 10 and 5 d. In addition, the bacterial density in the caeca has been observed to increase rapidly from hatch until 7 d, after which the density remains the same up to 30 d (Apajalahti et al., 2004). Similarly, the caecal microbial composition was observed to vary significantly in the first weeks of life (d 1 to d 10) but stabilised from 11 days on (Van Der Wielen et al., 2002). So, the seemingly longer retention of substrates in the caeca of younger birds is possibly a physiological adaptation to the underdeveloped caecal fermentation capacity early in life. Despite the higher Cr content in the caeca at a younger age compared to an older age at time points 3 and 5 h, the caecal influx ratio of Cr calculated 1 h after bolus administration did increase with age from 0.3% to 4% on d 9 to 0.8% to 75% on d 29. This indicates a higher influx of the liquid fraction from the ileum into the caeca at an older age, if caecal accumulation of the marker can be neglected at the 1 h time point. This age effect correspond to the trend observed by Vergara et al. (1989b) using the same marker. The caecal influx ratio of the cellulose beads was not affected by age, possibly due to the large biological variation, caecal accumulation of the beads over time or the different physiochemical characteristics of the beads compared to digesta particles as described above. In conclusion, the results indicate that caecal influx of the soluble fraction can increase with age, whereas the accumulation of both the solid and soluble fraction in the caeca seems greater at a young age (d 8 or 9) compared to an older age (d 29 or 36). This accumulation effect might be attributed to the underdeveloped caecal fermentation capacity very early in life. The addition of more accessible fibre adapted to the age-specific caecal fermentation capacity, especially at very young ages, might further improve the nutritious value of broiler feeds as the increased caecal retention time of substrates at this young age provides a longer period for potential microbial fermentation and thus enables a potentially beneficial evolution of the caecal microbiome.

The wide ranges of the caecal content and the caecal influx of both the solid and soluble markers demonstrate the high biological variation that seems to be inherent to studying the caecal influx mechanism in broilers. A possible cause is the complex caecal filling and emptying mechanism, which is still not fully understood. As the caeca are expected to fill and empty in cycles, it is possible that the asynchronisation of these cycles between broilers contributed to the biological variation measured during these types of marker studies despite the synchronised light regimes that were applied for all broilers in the current study (Clench and Mathias, 1999; Fenna and Boag, 1973). As the main objective of this research was to study the caecal influx mechanisms, no fasting was applied prior to

the marker administration in the current trials, as this may introduce a caecal filling and emptying behaviour that is atypical of the normal circumstances in which broilers are commercially raised. However, fasting the broilers before marker administration may contribute to the synchronisation of the marker movements along the GIT. This is common practice when using (fluorescent) markers in animals and would also decrease the issue caused by digesta autofluorescence, improving the detection of the fluorescence polystyrene beads (Li et al., 2018; van der Sluis et al., 2009). Another strategy to increase the synchrony in feeding patterns and, thus, caecal cycles between broilers is the use of a restricted feeding regime instead of continuous feeding or the adjustment of the sampling time points to the light regime. Both fasting and restricted feeding can provoke important changes in bowel motility, which is believed to also affect caecal filling, emptying and transit time, and were therefore not applied in the current study (Son et al., 2020).

5. Conclusion

This study showed that the caecal dimensions and digesta D50 increase with broiler age, suggesting that the digesta particle size allowing caecal influx also increases with age. A dense villous network at the entrance of the caeca restricts the inflow of digesta coming from the ileum, which explains the strong decrease in D50 of caecal digesta compared to ileal digesta. The effect of broiler age on the caecal filtering mechanism could be seen in the increased caecal diameter and digesta D50 with age on the one hand, but also in the increased caecal retention of markers at a young age compared to an older age on the other hand. The studied caecal digesta particle size distribution and influx of solid and soluble markers suggest that the caecal influx of particles larger than 100 μm is very limited. The content of the soluble marker Cr-EDTA in the caeca was maximally 13.2% of the initial orally administered dose in this study, illustrating the strong restriction of caecal influx for both solubles and solids. The caecal concentration of all three marker types showed considerable biological variation, which can be due to asynchronised caecal cycles between broilers. The study of caecal influx of markers was further complicated by the infrequent caecal filling and emptying dynamics which are still not fully understood, and by the possible accumulation of the used markers in the caeca. The results of this study highlight the physical limitations of caecal influx dictated by initial particle size and broiler age. Hence, carefully selecting the physical properties of fermentable fibre in function of age might aid in further improving caecal fibre fermentation in broilers.

Credit Author Statement

Paulien Vanderghinste: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **An Bautil:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Michael R. Bedford:** Writing – review & editing, Conceptualization. **Gemma González-Ortiz:** Writing – review & editing, Conceptualization. **Chris Lamberigts:** Methodology, Investigation. **Imran Aslam:** Resources, Methodology, Investigation. **Maarten Roeffaers:** Resources, Methodology. **Christophe M. Courtin:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

Colleagues of the Laboratory of Food Chemistry and Biochemistry, the Nutrition & Animal Microbiota EcoSystems Laboratory and the Laboratory of Livestock Physiology are thanked for their helping hands during the broiler trials. Marie Huyskens is thanked for performing the ICP-MS measurements. Quinten Wouters is thanked for assistance with the Leica TCS SP8X microscope. Paulien Vanderghinste acknowledges the Research Foundation Flanders for a position as PhD fellow (SB3423N).

Data availability statement

All data will be available in a data repository at https://doi.org/10.48804/SO5F0G upon publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.12.004.

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