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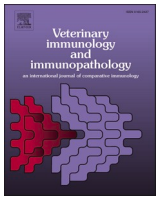

Influence of vitamin D metabolites on vitamin D status, immunity and gut health of piglets

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ABSTRACT

Immediately post-weaning, piglets are prone to gastrointestinal infectious diseases. The active metabolite of vitamin D 1,25-dihydroxyvitamin D has direct impact on immune cell function and responses. Thus, a low vitamin D status may compromise the immune responses during infectious diseases. The aim of this study was to examine the effect of supplementation of different forms of vitamin D (25-OH-D_3 and vitamin D_3) to suckling piglets' vitamin D status at weaning. In addition, to determine whether the vitamin D status could affect the immune development in piglets and their robustness against *E. coli* challenge. Genetically *E. coli* F4 susceptible litters of piglets were divided into two treatment groups: group 1 (n = 16) provided milk formula supplemented with vitamin D_3 (CON), and group 2 (n = 16) provided milk formula supplemented with 25-OH-D_3 (TREAT). Piglets were offered the experimental milk formulas from day 3 after farrowing until weaning (at day 28 of age). A commercial weaner diet with high protein content were provided to induce weaning stress. Milk formulas, sow and weaner diets as well as plasma and milk samples obtained from sows (n = 8) were analysed for vitamin D metabolites. Vitamin D status in piglets was investigated by collection of plasma samples on day 3, 15, 28 and 35 of age. Eight piglets randomly selected from each dietary group (in total 16 pigs) were inoculated with *E. coli* F4 O149 on day 2 and 3 post-weaning. Blood samples collected on day 2 and 9 post-weaning (pre- and post *E. coli* inoculation, respectively) were analysed for haematological and immunological parameters including immunoglobulins, antibodies specific to *E. coli* O149 K88, cytokines and C-reactive protein. In addition, intestinal samples were obtained one week after *E. coli* inoculation to study the influence of infection and vitamin D status on immune responses at different sites of the intestine. This was accomplished by gene expression of various cytokines and tight junction proteins. In general, vitamin D status of the piglets were low. However, piglets provided TREAT during the suckling period had increased vitamin D status at weaning compared to piglets provided CON. Vitamin D was used during activation of the immune system as pigs inoculated with *E. coli* had lower plasma concentrations of 25-OH-D_3 than non-inoculated pigs possibly due to mobilising of vitamin D in the liver. Hence, increased vitamin D status at weaning might improve piglets' resistance to *E. coli* infection.

1. Introduction

Vitamin D status in humans and animals is measured as the concentration of 25-OH-D_3 in plasma, and among livestock species, piglets are born with the lowest vitamin D status, below 4 ng 25-OH-D_3/mL plasma (Horst and Littledike, 1982). Studies have reported that the transfer of fat-soluble vitamins, including vitamin D, is restricted during gestation probably due to the epitheliochorial nature of the porcine placenta, which limits the transfer of fatty acids and fat-soluble vitamins from sow to foetuses (Ramsay et al., 1991; Père, 2003). Furthermore, it has been suggested that sow colostrum and milk are not sufficient to fulfil the vitamin D requirements of piglets (Matte and Audet, 2020).

Abbreviations: Co, colon; CRP, C-reactive protein; HCT, haematocrit; HGB, haemoglobin; IgG, immunoglobulin G; NLR, neutrophils to lymphocytes ratio; PP, Peyer's patches; SCFA, short chain fatty acid; Si, small intestine.

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Vitamin D_3, which is the traditional form of vitamin D supplementation for pig nutrition, is after absorption transported via the circulating blood to the liver where it is hydroxylated to 25-OH-D_3, which is further metabolised to 1α,25-dihydroxyvitamin D_3 mainly in the kidney by 1α-hydroxylase. The metabolite 25-OH-D_3 is the major circulating metabolite of vitamin D and is the most reliable measurement of the vitamin D status of an individual, while 1α,25-dihydroxyvitamin D_3 is the bioactive form of vitamin D, which plays a role in the immune system, but its concentration is difficult to measure (Aranow, 2011). In addition, 25-OH-D_3 can also be converted to 24,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ by the CYP24A1 enzyme (Jones et al., 2012). The vitamin D metabolite 25-OH-D_3 is available in a commercial form as HyD® (DSM Nutritional Products). This metabolite is considered to be an optimal dietary source of vitamin D for pigs including piglets, as it has shown to increase serum 25-OH-D_3 levels compared to vitamin D_3, when both D_3 and 25-OH-D_3 were provided at same amounts for sows (Lauridsen et al., 2010). In addition, Weber et al. (2014) reported increased plasma concentration of 25-OH-D_3 in sows receiving 25-OH-D_3 when compared to provision of vitamin D_3, which might be due to the higher bioavailability of 25-OH-D_3 than the traditional vitamin D_3 (Schuster, 2011).

It is well established that vitamin D plays an important role in calcium and bone metabolism (Lips and van Schoor, 2011; Bikle, 2012). More recently, especially in relation to human health, the role of vitamin D in the immune system has been revealed (Baeke et al., 2010a; Maruotti and Cantatore, 2010; Chun et al., 2014). Several studies have reported that 1α,25-dihydroxyvitamin D_3 is an important mediator of intestinal epithelial defences against pathogens by regulating tight junction molecule expression and intestinal barrier function (Kong et al., 2008; Assa et al., 2014). In addition, 1α,25-dihydroxyvitamin D_3 is an important regulator of the innate immune responses as it enhances the antimicrobial properties of immune cells including monocytes and macrophages (Baeke et al., 2010b). Furthermore, it has been demonstrated that recognition of lipopolysaccharides through toll-like receptors leads to increased binding of 1α,25-dihydroxyvitamin D_3 to vitamin D receptors and thus an upregulation of the transcription of antibacterial peptides such as cathelicidin and beta defensin 4 (Liu et al., 2006). Expression of antimicrobial peptides such as cathelicidin is induced by 1α,25-dihydroxyvitamin D_3 in myeloid cells as well as in epithelial cells including the epithelium of the intestine (Bikle, 2008). Deficiency in 25-OH-D_3 or lack of vitamin D receptors inhibit the ability of epithelial cells to produce cathelicidin in response to a microbial challenge (Wang et al., 2004; Liu et al., 2006). Individuals with vitamin D deficiency, characterised by a low plasma concentration of 25-OH-D_3 might be less able to produce cathelicidin and therefore may be at greater risk of infection including infections in the gastrointestinal tract (Bikle, 2008). Taken together it is clear from the literature that vitamin D mediates the regulation of the intestinal epithelium and mucosal immune system that shape the microbial communities in the gut to maintain homoeostasis (Cantorna et al., 2019). Thus, increasing the vitamin D status in suckling piglets may influence the intestinal immune system and the microbial function and might reduce the incidence of enteric infections leading to post-weaning diarrhoea, and may therefore be considered as an alternative strategy to antibiotics and medical zinc-oxide as recently proposed by Lauridsen et al. (2021).

Because the commercial management of large litters of suckling pigs includes provision of milk formulas as a nutritional supplement to the sow milk, milk formulas may form a vehicle for the transfer of vitamin D and thus a strategic tool of enhancing the vitamin D status at weaning in piglets. However, yet no studies have been conducted in dams and piglets to study the impact on vitamin D status and immunological responses and the function of the enteric microbiome. Therefore, we hypothesised that provision of milk formula supplemented with different forms of vitamin D including 25-OH-D_3 and vitamin D_3 to piglets during the suckling period influences vitamin D status at weaning. In addition, it is hypothesised that the vitamin D status at weaning will affect haematological, immunological responses and microbial metabolites measured in plasma and at various sites of the intestine in piglets when challenged with *E. coli* post-weaning.

2. Materials and methods

The animal experiment was carried out according to the license (# 2017–15–0201–01270) obtained from the Danish Animal Experiments Inspectorate, Danish Veterinary and Food Administration, Ministry of Food, Agriculture and Fisheries.

2.1. Experimental design

Genetically homozygous *E. coli* F4 susceptible sows (n = 8) from the herd at AU Viborg Research Centre Foulum, Aarhus University, were used in the experiment to breed *E. coli* F4 susceptible piglets (101 piglets were born in total). The sows and their litters (4 piglets from each sow selected based on sex and body weight) were divided into two groups: group 1 (n = 16) consisted of 4 litters, which were provided milk formula supplemented with vitamin D_3 (CON) and group 2 (n = 16) consisted of the remaining 4 litters, which were provided milk formula supplemented with 25-OH-D_3 (TREAT). The piglets were provided the experimental milk formulas from day 3 of age until the day of weaning (at day 28 of age). On day 2 and 3 post-weaning, 8 pigs were randomly selected from each dietary treatment group and were inoculated with *E. coli* F4 O149. Blood samples were collected from all pigs at day 2 and 9 post-weaning (pre- and post *E. coli* inoculation, respectively) for analysis of immunological parameters. In addition, intestinal samples were obtained one week after *E. coli* inoculation (10 days post-weaning) to study the influence of infection and vitamin D status on intestinal immune responses.

2.2. Animals, housing and feeding

Sows (Danish Landrace x Yorkshire x Duroc) from the AU Viborg - Research Centre Foulum were mated with Danish Landrace and enroled in the experiment one week before farrowing. The windows in the barn (AU Viborg Research Centre Foulum) were covered with dark plastic cover to prevent sunlight exposure to the animals in order to avoid vitamin D generation via external sources. Piglets were ear-labelled at birth. At day 2 of age, piglets received an injection with iron-dextran, were tail-docked and all male piglets were castrated, and cross fostering of piglets within sow treatment groups was performed to ensure equal numbers of piglets per litter. From day 3 after farrowing and until weaning at day 28 of age, piglets were in addition to sow milk offered milk formula treatments according to the dietary treatments CON and TREAT. The milk formula was provided via an automatic system (Babydos Bopil, Sønderborg, Denmark) in a trough (semicircle with radius of 10 cm) and needed no activation from the piglets. The supplemental milk was accessible ad libitum. At weaning, the pigs selected for *E. coli* inoculation (n = 16) were housed pair-wise with littermates in a pen (non-litter mates were not mixed within pens). The pigs were all offered the same weaner diet with a high protein content to induce weaning stress, and they continued immediately after weaning from the dam in the *E. coli* challenge as described in detail below. The two remaining piglets from each litter were not inoculated with *E. coli*, and continued in a separate trial carried out in the same barn facility at AU Viborg - Research Centre Foulum, but in a different room to avoid contamination from *E. coli* infected pigs and hence functioned as a control group (n = 16).

2.3. Diets

Piglets were in addition to sow milk offered the milk formula treatments according to the dietary treatments CON and TREAT from day 3 after farrowing and until weaning at day 28 of age. The experimental milk formulas were manufactured by Schils, the Netherlands, and

provided by DSM Nutritional Products, Kaiseraugst, Switzerland, and contained the commercial forms CON (Rovimix D3) and TREAT (Rovimix HyD®) containing per kg feed 4999 IU of vitamin D in the form of vitamin D_3 and 25-OH-D_3, respectively. At weaning, the pigs were all offered the same weaner diet mixed at AU Viborg - Research Centre Foulum (Supplementary file Table 1), which was based on a high protein content and with no vitamin and mineral supplementation to avoid additional vitamin D supplements. Sows were allocated a standard Danish sow lactation diet provided from DLG, Denmark, which contained 25-OH-D_3 as the sole vitamin D source (2000 IU per kg; Supplementary file Table 1). The two milk formulas, the weaner diet and the sow lactation diet were analysed for major nutritional composition at Eurofins Steins Laboratory A/S, and vitamin D content including vitamin D_3 and 25-OH-D_3 was analysed at DSM Nutritional Products, Kaiseraugst, Switzerland.

2.4. Sampling and recordings

Plasma and milk samples were obtained from the sows (n = 8) on day − 7 (day 108 of gestation; plasma samples) and on day 3, 14 and 28 of lactation (plasma and milk samples; Fig. 1). Four medium-weight piglets per litter were selected on day 3 for collection of plasma on day 3, 15 and 28 of the suckling period. All samples were stored at − 20 °C until analysis of vitamin D content including concentration of 25-OH-D_3 for assessment of vitamin D status. Piglets were individually weighed at day 3 of age, and each litter was weighed weekly until weaning where they were all individually weighed again. Health status of the animals was recorded daily, and dead pigs were noted as well as any medical treatment. At weaning, two of the four piglets from each litter, which were used for blood sampling during suckling, were selected and weaned on day 28 of age and were used in a trial with *E. coli* inoculation (n = 16). On day 2 and 3 post-weaning, the pigs (n = 16; 8 pigs from CON and 8 pigs from TREAT) were inoculated with *E. coli* O149 K88 (2.32×10^9 and 1.32×10^9 CFU per pig on day 2 and 3 post-weaning, respectively). The two remaining piglets from each litter, which were used for blood sampling during suckling, were not inoculated with *E. coli*, but continued in a separate trial carried out in the same facility in a different room and hence functioned as a control group (n = 16). On day 35 of age (8 days post-weaning), plasma samples for vitamin D analysis were collected from the same four pigs which had been used for blood sampling during the suckling period (n = 32). On day 2 and 9 post-weaning (at day 29 and 36 of age, respectively), sodium-heparinized blood samples (4 mL/pig) were collected from the jugular vein of *E. coli* inoculated pigs (8 pigs from each dietary treatment group, i.e., in total 16 pigs) as well as from non-inoculated pigs (8 pigs from each dietary treatment group, 16 pigs in total).

Whole blood obtained from pigs on day 2 and 9 post-weaning (n = 64) was analysed for haematological parameters. Plasma was obtained after centrifugation at 3000 x g and subsequently stored at − 80 °C until analysis of immunological parameters. Before euthanization of the *E. coli* inoculated pigs on day 37 of age (10 days post-weaning), faeces samples were collected from all pigs in order to quantify *E. coli* as well as enterotoxins and the samples were stored at − 80 °C until analysis. After the pigs were euthanized by using blunt trauma, the sampling was focused on the immune system and epithelial barrier function to investigate the effect of vitamin D treatment during suckling on gut immune responses after *E. coli* inoculation. Organs including liver, heart and lungs were weighed, and the intestines were measured and divided into small intestine (Si; 50% (Si50) of length), caecum and colon (Co; 50% (Co50) of length). Digesta samples from the stomach, Si, caecum and Co were collected in order to analyse short chain fatty acid (SCFA) content. In addition, mucosal samples were collected from two sites of the Si, pre- and post-Peyer's patches (PP), respectively, in order to analyse mucosal IgA content. Furthermore, intestinal samples including Si50 and CO50 as well as mucosal scrapings (pre-PP and post-PP) were collected for gene expression analysis. All intestinal and digesta samples were stored at − 80 °C until analysis.

2.5. Laboratory analyses

2.5.1. Analysis of vitamin D metabolites

Samples of the diets obtained from the sow lactation diet, the two milk formulas and weaner diet as well as samples of plasma from sows (n = 32), sow milk (n = 24) and plasma from pigs (n = 128) were analysed at DSM Nutritional Products, Kaiseraugst, Switzerland, for vitamin D content including vitamin D metabolites. The content of vitamin D_3 in the milk formulas as well as the sow and weaner diets were analysed by addition of an internal standard followed by saponification of the sample with potassium hydroxide alkaline ethanol solution and extraction with cyclohexane. Subsequently, vitamin D_3 was quantified by a reversed-phase HPLC-MS/MS method. The quantification was carried out by using 6,19,19-trideutero-vitamin D_3 as internal standard. Quantification of 25-OH-D_3 in the milk formulas as well as the sow and weaner diets was analysed by addition of an internal standard, saponification of the sample and subsequently extraction of 25-OH-D_3 with tert-butyl methyl ether. The extract was dried by evaporation and then analysed after solubilisation by reversed phase high-performance liquid chromatography with MS/MS detection. The quantification was carried out by using labelled deuterium-6–25-hydroxy cholecalciferol as internal standard.

The plasma and sow milk samples were analysed for vitamin D_3 and its main known metabolites (24(R),25-dihydroxyvitamin D_3, 25-OH-D_3, 3-epi-25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2) by addition of an internal standard to an aliquot of plasma or milk followed by

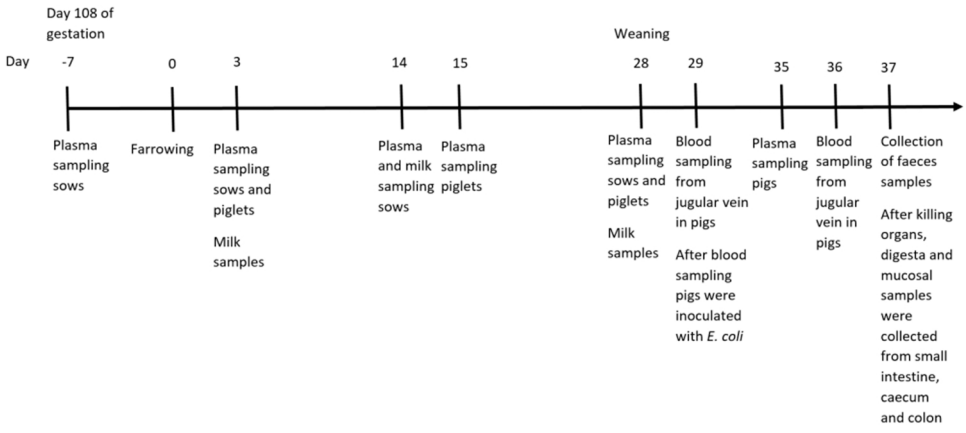

Fig. 1. Overview of collection of samples during the experiment.

extraction by protein precipitation with acetonitrile. After centrifugation and filtration, the supernatant was evaporated and the residue was reconstituted with methanol-acetonitrile-water solution. Analysis was done by a reverse phase chromatography coupled with mass spectrometry detection. Quantification was done by applying dedicated external calibrations (using deuterated internal standards). To assess the daily and long-term laboratory performance (accuracy and precision) of the method, standard and quality control samples were analysed daily with blind samples.

2.5.2. Analysis of immunological parameters

Immediately after collection of blood samples from pigs on day 2 and 9 post-weaning (n = 64), analysis of whole blood was performed as a diagnostic health measurement using a haematology analyser (IDEXX ProCyte Dx®). Haematological parameters were total leucocytes, neutrophils, lymphocytes, neutrophils to lymphocytes ratio (NLR), monocytes, eosinophils, haemoglobin (HGB), haematocrit (HCT), the mean cell volume, the mean corpuscular haemoglobin and the mean corpuscular haemoglobin concentration. In addition, the blood samples from day 2 and 9 post-weaning (n = 64) were analysed for the following immunological parameters: immunoglobulin G (IgG), antibodies specific to *E. coli* O149 K88, cytokines and C-reactive protein (CRP). Quantification of IgG in plasma samples was done using an ELISA kit (Abnova, Bio-Rad, Richmond, CA, USA) according to manufacturer's instructions. The level of specific antibody reactivity against *E. coli* O149 K88 in plasma was performed using ELISA as described by Lauridsen and Jensen (2005). The following cytokines: IFN-γ, TNF-a, IL-1α, IL-1β, IL-1RA, IL-4, IL-6, IL-8, IL-10, IL-12 and IL-18 were quantified by Multiplex (Procarta Porcine Cytokine Assay Kit, Panomics,CA, USA) on a Luminex 100 (Bio–Rad, Richmond, CA, USA). Finally, plasma samples were analysed for CRP concentration by solid-phase sandwich immunoassay using a Phase Porcine CRP Assay Kit according to manufactory guidelines (Tridelta Development Ltd., Kildare, Ireland). Mucosal samples from Si were analysed for concentration of IgA using commercially available sandwich ELISA kit (Abnova), and the concentration of mucosal IgA was expressed as the relative amount of IgA to total protein. Total protein content in mucosa was analysed on an autoanalyser, OpeRA™, Chemistry System (Bayer Corporation) according to the biuret method as standardised by the Technicon RA® Systems (Bayer Corp., 1994). Intestinal content including digesta from the stomach, Si, caecum and Co was analysed for SCFA content according to the procedure described by Canibe et al. (2007).

2.5.3. Quantification of E. coli and enterotoxins in faeces

Determination of *E. coli* and enterotoxins in faeces samples was accomplished by quantification of genes encoding *E. coli* (ybbW) and the enterotoxins LT2 (eltB) and STb (est B) by qPCR. Standard curves were obtained from counted reference strain for *E. coli* AUF4 (9910045–1) spiked into faeces from a healthy pig with no background ETEC F4. Faeces samples were defrosted (~50 mg) for DNA extraction and purification using NucleoSpin 96 Stool DNA kit (Machery Nagel, Düren, Germany) following the manufacturer's directions with the following modifications. Samples were homogenised by addition of SLX buffer in a Star-beater (VWR) at frequency 30 (1/s) for 5 min, and DNA was eluted in 150 μL of elution buffer. Subsequently, DNA concentration was determined using a Qubit Fluorometer. Quantitative real-time PCR was performed on a ABI ViiA7™ Real-Time PCR System (Thermo Fisher Scientific) using MicroAmp Optical 384 well reaction plate (Applied Biosystems). Quantitative real-time PCR reactions contained 5 μL of RealQ Plus Mastermix (Amplicon), primers for *E. coli* and enterotoxins at a concentration of 0.3 mM, and 2 μL of template DNA and water to a total volume of 10 μL. Quantification was determined with the following programme: pre-treatment for 2 min at 50 °C followed by initial denaturation (10 min at 95 °C) and subsequently 40 cycles of denaturation for 15 s at 95 °C, 30 s for primer annealing at different temperatures (Supplementary file Table 2) and 30 s at 72 °C for base extension. Melting curves were derived by increasing the temperature from 60 to 95 °C at a rate of 0.05 °C/s, recording continuously. These curves were used to evaluate the quality of the PCR products. All analyses were performed in triplicate. Target concentrations were calculated using the QuantStudio Real-Time PRC software from the standard curve. The limit of quantification was set at Ct values greater than 32, which corresponded to 10^5 cells/g faeces.

2.5.4. Gene expression in intestinal and mucosal samples

Expression of genes in intestinal and mucosal samples was analysed with high-throughput qPCR using a 192.24 Dynamic Array Integrated Fluidic Circuit chip (Fluidigm, San Francisco, CA, USA) according to Skovgaard et al. (2013) with minor modifications including 18 cycles of pre-amplification. Initially, total RNA (500 ng) was extracted from the samples using a NucleoSpin RNA kit (Ref. 740955 Macherey-Nagel, Germany) including DNAse treatment followed by synthesis of complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Ref. 4368813, Applied Biosystems, USA) according to manufactory instructions. A list of gene names, mRNA primer sequences and amplicon length are included in a separate file (Supplementary file Table 3). Data (Vq values) were obtained using the Fluidigm Real-Time PCR Analysis software 3.0.2 (Fluidigm, San Francisco, CA, USA) and exported to GebEx6 (MultiD) for data processing including correction for PCR efficiency and normalisation to reference genes. Using geNorm and NormFinder algorithms, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Peptidylprolyl Isomerase A (PPIA) and TATA-Box Binding Protein (TBP) were selected as reference genes and used for data normalisation. During transformation from log2 to linear scale, the sample with the lowest mean mRNA expression was assigned the value of 1 for each gene, and relative expressions of the remaining samples were calculated according to this.

2.6. Statistical analyses

All statistical analyses were performed in R version 4.1.0 (R Core Team, 2021). The impact of dietary treatments (CON vs TREAT) and age (day 3, 15 and 28) on plasma concentrations of 25-OH-D_3 and 24(R), 25-$(OH)_2$-D_3 was examined by a linear mixed effects model using the *lme* function from the *nlme* package (Equation 1):

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \omega_k + \varepsilon_{ijkl}$$

where Y_{ijkl} is the log-transformed dependent variable, μ is the overall mean, α_i is the dietary treatment (i = CON or TREAT), β_j is the age (j = day 3, 15 or 28), $\alpha\beta_{ij}$ is their interaction, $\omega_k \sim N(0, \sigma_\omega^2)$ is the random effect of litter k, $\varepsilon_{ijkl} \sim N(0, \sigma^2)$ is the residual error and $l = 1, 2, \ldots, n_{ik}$ is piglets within litter k receiving treatment i. In addition, an auto regressive process of order 1 (AR(1)) covariance structure was used to model the correlation between repeated measurements within piglet, i.e. $cor(\varepsilon_{ij_1kl}, \varepsilon_{ij_2kl}) = \rho^{|j_1 - j_2|}$. Moreover, BW of the piglets at birth, at day 14 of age and at weaning (at day 28 of age) was analysed according to Equation 1.

The impact of dietary treatments (CON vs TREAT) and group (*E. coli* inoculated pigs vs non-inoculated pigs) on plasma concentrations of vitamin D metabolites at day 35 of age, immunological and haematological parameters on day 9 post-weaning, and faecal shedding of *E. coli* and enterotoxins was analysed according to the following model (Equation 2):

$$Y_{idkm} = \mu + \alpha_i + \gamma_d + \alpha\gamma_{id} + \omega_k + \varepsilon_{idkm}$$

where Y_{idkm} is the log-transformed dependent variable, μ is the overall mean, α_i is the dietary treatment (i = CON or TREAT), γ_d is the group (d = *E. coli* inoculated pigs or non-inoculated pigs), $\alpha\gamma_{id}$ is their interaction, $\omega_k \sim N(0, \sigma_\omega^2)$ is the random effect of litter k, $\varepsilon_{idkm} \sim N(0, \sigma^2)$ is the residual error and $m = 1, 2 \ldots, n_{idk}$ is piglets in group d from litter k

receiving treatment *i*. In addition, immunological and haematological parameters on day 2 post-weaning were analysed according to Equation 2 without group as a factor in the model. Furthermore, the effect of dietary treatment and sample site on mucosal IgA concentration, SCFA concentration in digesta and gene expression (qPCR data) was analysed according to Equation 2 with sample site as a factor in the model. To approach a normal distribution, qPCR data were log2transformed.

Regarding the sows, plasma concentrations of vitamin D metabolites on day − 7, 3, 14 and 28 of the suckling period were examined by the following model using the log-linked Gamma function (Equation 3):

$$Y_{io} = \mu + \delta_i + \omega_o + \varepsilon_{io}$$

where Y_{io} is the log-transformed dependent variable,μ is the overall mean, δ_i is the day of the suckling period (i = day −7, 3, 14 or 28), $\omega_o \sim N(0, \sigma^2_\omega)$ is the random effect of sow o and $\varepsilon_{io} \sim N(0, \sigma^2)$ is the residual error.

For all models (Equation 1, 2 and 3), estimated least squares means (LS means) ± SEM were obtained using the *emmeans* package. Exploratory comparisons were carried out with emmeans on log-scale. Differences were considered significant when $p < 0.05$ and trends when $p < 0.10$.

3. Results

Body weight of the piglets was not affected by milk formula treatment ($p \geq 0.12$), but increased during the suckling period ($p < 0.001$; data not shown).

3.1. Vitamin D content in diets

The analysed concentration of vitamin D metabolites as well as other major nutrients in the sow lactation diet, the experimental milk formulas and the weaner diet are presented in Table 1.

The analysed vitamin D content of the sow diet, milk formulas and weaner diet corresponded to the expected values, except that the milk formula supplemented with vitamin D_3 also contained 25-OH-D_3 (40% and 60% of 25-OH-D_3 and vitamin D_3, respectively). The total content of vitamin D in the milk formulas were 4424 IU for milk formula CON and 4480 IU for milk formula TREAT. Vitamin D was not detected in the weaner diet, which was in accordance with our planned design.

3.2. Vitamin D content in sow plasma and milk

The plasma concentration of 25-OH-D_3 in sows decreased post farrowing, followed by an increase on day 14 during the suckling period, and subsequently decreased on day 28 of the suckling period ($p < 0.02$; Table 2). The plasma concentration of 25-OH-D_2 in sows had a numerical decrease from day 3 until day 14 of the suckling period, and subsequently increased from day 14 to day 28 of the suckling period ($p < 0.02$). The plasma concentration of 24(R),25-$(OH)_2$-D_3 in sows decreased post farrowing ($p < 0.01$) but appeared constant during the suckling period.

In sow milk, the concentration of vitamin D metabolites was below measurable amounts in all milk samples (n = 24) on day 3, 14 and 28 of the suckling period, i.e., the concentration of analysed 25-OH-D_3 in sow milk was detectable in only 6 out of 24 samples with concentrations slightly above 1.00 ng/mL [1.17 ± 0.46 ng/mL]. In addition, the concentration of vitamin D_3 was below 50.0 ng/mL, and the concentration of analysed 24(R),25-$(OH)_2$-D_3 was below 0.50 ng/mL.

3.3. Effect of milk formula treatment on vitamin D status in piglets

During the suckling period from day 3 to day 28 of age, the plasma concentration of 25-OH-D_3 increased in piglets allocated TREAT ($p < 0.001$) and tended to increase in piglets provided CON ($p = 0.07$; Fig. 2a). No difference was observed between milk formulas in the plasma concentration of 25-OH-D_3 on day 3 ($p = 0.99$) and 15 of age ($p = 0.10$). However, on day 28 of age, the plasma concentration of 25-OH-D_3 tended to be higher in piglets provided TREAT compared to piglets provided CON ($p = 0.07$). From day 3 to day 15 of age, the plasma concentration of 24(R),25-$(OH)_2$-D_3 decreased in piglets allocated CON ($p = 0.002$), whereas no difference was observed in piglets allocated TREAT ($p = 0.15$; Fig. 2b). From day 15 to day 28 of age, the plasma concentration of 24(R),25-$(OH)_2$-D_3 increased in piglets allocated TREAT ($p = 0.001$); however, no difference was observed in piglets allocated CON ($p = 0.28$). Furthermore, no difference was observed in the plasma concentration of 24(R),25-$(OH)_2$-D_3 between milk formulas on day 3 ($p = 0.63$) and 15 of age ($p = 0.27$). On day 28 of age, however, the plasma concentration of 24(R),25-$(OH)_2$-D_3 tended to be higher in piglets provided TREAT compared to piglets provided CON ($p = 0.05$).

3.4. Effect of E. coli inoculation on vitamin D status in pigs

On day 35 of age (7 days post *E. coli* inoculation), the plasma concentration of 25-OH-D_3 was reduced by 40.3% in *E. coli* inoculated pigs compared to non-inoculated pigs, when pigs were allocated CON during the suckling period ($p = 0.03$; Fig. 3a). A similar tendency was obtained when pigs were provided TREAT during the suckling period; i.e., on day 35 of age, the plasma concentration of 25-OH-D_3 was reduced by 35.4% after *E. coli* inoculation ($p = 0.14$). The plasma concentration of 24(R),25-$(OH)_2$-D_3 tended to be higher in *E. coli* inoculated pigs compared to control pigs allocated CON during the suckling period ($p = 0.05$; Fig. 3b). No difference was observed in the plasma concentration of 24(R),25-$(OH)_2$-D_3 between *E. coli* inoculated pigs and control pigs provided TREAT during the suckling period ($p = 0.95$).

Table 1
Analysed chemical composition of sow lactation diet, experimental milk formulas and weaner diet.

Item	Sow lactation diet	Milk formula CON	Milk formula TREAT	Weaner diet
DM, %	87.2	96.0	95.7	87.7
Crude protein, % of DM	17.8	20.3	20.4	26.8
Crude fat, % of DM	4.36	17.9	17.9	4.10
Crude ash, % of DM	5.62	8.96	8.99	5.82
Vitamin D_3, IU/kg	<LOD	2620	<LOQ	<LOD
25-OH-D_3, IU/kg	1480	1804	4480	<LOQ
EDOM[1], %	86.0	99.4	99.8	91.1
ME[2], MJ/kg in DM	15.1	21.9	21.9	15.6

[1] Enzyme digestibility of organic matter.
[2] Metabolisable energy: estimated mainly based on EDOM and results of crude fat and crude protein.

Table 2
Plasma concentrations (ng/mL) of vitamin D metabolites in sows.[1]

	Day[2]					
Item	-7	3	14	28	SEM[3]	p-value
25-OH-D_3	39.3^a	34.4^b	47.9^c	42.8^d	2.85	< 0.02
25-OH-D_2	0.70^a	0.68^a	0.63^a	0.98^b	0.05	< 0.02
24(R),25-$(OH)_2$-D_3	16.9^a	14.7^b	15.0^b	14.8^b	1.15	< 0.01

[1] Values are presented as LS means, n = 8. Mean values within a row with different superscript letters are significantly different.
[2] Blood samples were collected from sows at day 108 of gestation (7 days before farrowing) and at day 3, 14 and 28 during the suckling period.
[3]Average standard errors of LS means.

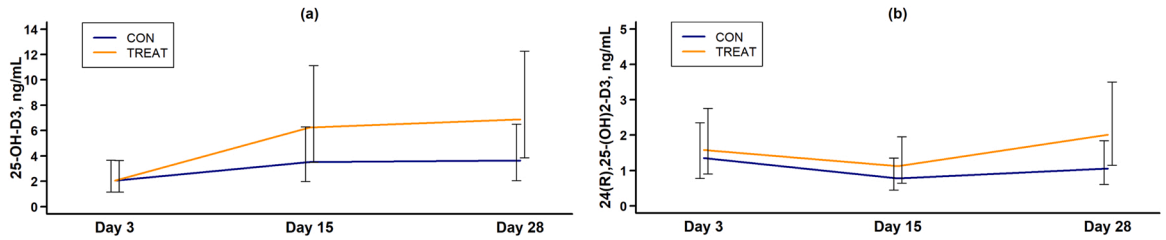

Fig. 2. Plasma concentration of 25-OH-D_3 (a) and 24(R),25-$(OH)_2$-D_3 (b) in piglets allocated CON or TREAT from day 3 of age until day 28 of age. Values are LS means, and vertical bars represent standard errors, n = 16.

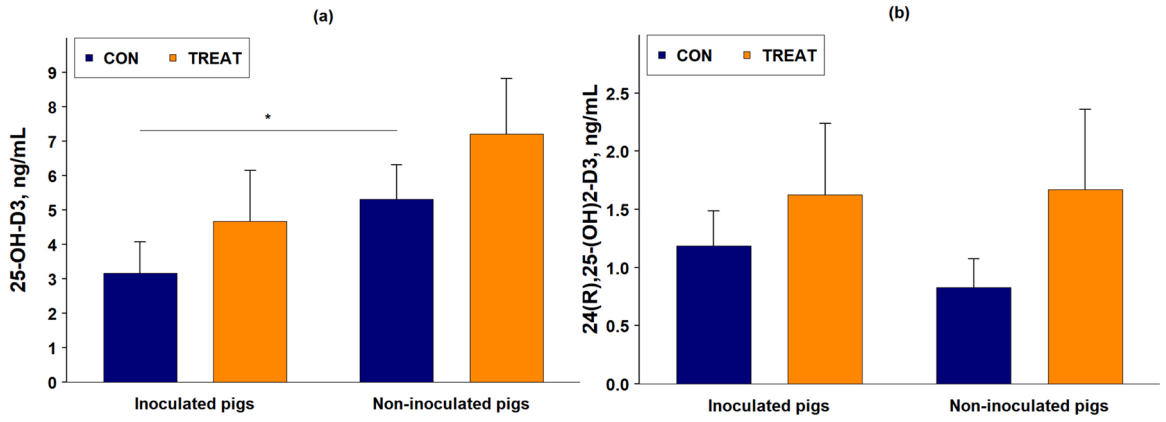

Fig. 3. Plasma concentration of 25-OH-D_3 (a) and 24(R),25-$(OH)_2$-D_3 (b) in *E. coli* inoculated pigs and non-inoculated pigs on day 35 of age (8 days post-weaning) allocated CON or TREAT during the suckling period. Values are LS means, and vertical bars represent standard errors, n = 16.

Furthermore, the plasma concentration of vitamin D_3 in piglets was detectable in only 23 out of 128 samples on day 15, 28 and 35 post farrowing with concentrations slightly below 1.00 ng/mL [0.90 ± 0.44 ng/mL].

3.5. Quantification of *E. coli* and enterotoxins in faeces

The number of *E. coli* and the enterotoxins LT and STb2 were higher ($p < 0.001$) in faeces of *E. coli* inoculated pigs compared to control pigs (Table 3). No difference was observed between pigs provided CON or TREAT during the suckling period ($p \geq 0.06$).

3.6. Immunological responses

3.6.1. Haematological parameters, cytokines and CRP

Haematological parameters as well as plasma concentrations of cytokines and CRP in pigs on day 2 post-weaning before inoculation with *E. coli* and on day 9 post-weaning after *E. coli* inoculation are shown in Tables 4 and 5, respectively. On day 2 post-weaning, pigs provided TREAT had higher levels of neutrophils, NLR, IFN-γ and TNF-α as well as lower levels of lymphocytes than pigs provided CON during the suckling period.

On day 9 post-weaning, pigs inoculated with *E. coli* had lower levels of monocytes, eosinophils, IFN-γ and CRP than non-inoculated pigs. Furthermore, an interaction between milk formula treatment and *E. coli* inoculation was observed for plasma concentration of IFN-γ, TNF-α and CRP.

3.6.2. IgA mucosal concentration in *E. coli* inoculated pigs

No differences in mucosal IgA concentrations were observed between milk formula treatments in *E. coli* inoculated pigs (Table 6). However, sample site had an effect on the IgA concentration, i.e., the concentration of IgA was higher in samples collected post-PP than in samples collected pre-PP.

3.6.3. IgG and specific antibody responses

For both *E. coli* inoculated pigs and non-inoculated pigs, the plasma concentration of IgG increased from day 2 to day 9 post-weaning (7 days post *E. coli* inoculation) irrespectively of the milk formula treatment provided during suckling ($p < 0.001$). In addition, pigs provided CON had higher plasma concentrations of IgG than pigs provided TREAT (Fig. 4a). No difference was observed between *E. coli* inoculated pigs and non-inoculated pigs, however, in samples collected on day 9 post-weaning, the plasma concentration of IgG tended to be higher in

Table 3
Quantification of *E. coli* and enterotoxins in faeces[1,2] of *E. coli* inoculated pigs and control pigs provided CON or TREAT during the suckling period.

Group	*E. coli* inoculated pigs		Control pigs		SEM[3]	p-value	
Treatment	CON	TREAT	CON	TREAT		Treatment	Group
E. coli	8.49	8.40	6.67	6.84	6.07	0.06	< 0.001
LT	8.06	8.56	3.55	3.74	6.11	0.33	< 0.001
STb2	8.44	8.52	6.67	6.68	6.14	0.22	< 0.001

[1] Faeces samples were collected on day 37 of age (10 days post-weaning), n = 8.
[2] Log10 copies/g sample.
[3] Average standard errors of LS means.

Table 4
Haematological parameters as well as plasma concentrations of cytokines and CRP in pigs on day 2 post-weaning provided CON or TREAT during the suckling period.[1]

Treatment	CON	TREAT	SEM[2]	p-value
WBC[3], g/L	13.8	12.4	0.72	0.21
Neutrophils, %	31.7	40.6	1.90	< 0.01
Lymphocytes, %	62.6	54.3	1.96	0.01
NLR[4]	0.54	0.77	0.06	< 0.01
Monocytes, %	4.94	4.46	0.32	0.29
Eosinophils, %	0.73	0.59	0.11	0.89
HGB[5], g/L	96.8	108	4.54	0.09
HCT[6], %	32.0	36.0	1.52	0.07
MCV[7], x10^{-15} L	55.0	59.3	1.85	0.11
MCH[8], pg/mL	16.6	17.8	0.55	0.14
MCHC[9], g/L	303	300	2.52	0.46
IFN-γ, ng/mL	12.6	20.2	2.38	0.03
IL-1α, ng/mL	0.014	0.008	0.003	0.27
IL-1β, ng/mL	0.10	0.27	0.08	0.39
IL-1RA, ng/mL	0.47	0.36	0.07	0.21
IL-4, ng/mL	0.19	0.08	0.05	0.10
IL-6, ng/mL	0.038	0.027	0.008	0.42
IL-8, ng/mL	0.03	0.04	0.005	0.10
IL-10, ng/mL	0.13	0.07	0.02	0.06
IL-12, ng/mL	0.84	0.92	0.08	0.45
IL-18, ng/mL	0.40	0.36	0.06	0.27
TNF-α, ng/mL	0.03	0.08	0.01	< 0.01
CRP, μg/mL	203	167	43	0.55

[1] Blood samples were obtained 2 days post-weaning (on day 29 of age). Values are LS means, n = 16.
[2] Average standard errors of LS means.
[3] WBC white blood cells.
[4] NLR neutrophils to lymphocytes ratio.
[5] HGB haemoglobin.
[6] HCT haematocrit.
[7] MCV the mean cell volume-the ratio of the haematocrit to the concentration of red blood cells.
[8] MCH the mean corpuscular haemoglobin-the ratio of the total mass of haemoglobin to the number of red blood cells.
[9] MCHC the mean corpuscular haemoglobin concentration – identifies the amount of haemoglobin in a single red blood cell.

E. coli inoculated pigs than in non-inoculated pigs allocated CON during the suckling period ($p = 0.09$). Pigs allocated CON had higher plasma concentrations of specific antibodies against *E. coli* F4 compared to pigs allocated TREAT both before and after inoculation with *E. coli* F4, and in non-inoculated pigs on day 9 post-weaning (Fig. 4b). In addition, from day 2–9 post-weaning the concentration of specific antibodies increased in *E. coli* inoculated pigs provided TREAT ($p < 0.001$).

3.7. Gene expression

Expression of genes in the intestine was not affected by milk formula treatments ($p > 0.10$) but was highly affected by segment of the intestine ($p < 0.05$; Fig. 5). Genes coding for the following cytokines: IL1RAP, IL18, IL12B, IL10, IL8 and IL1B were upregulated ($p \leq 0.01$) in Si50 compared to Co50. In addition, the gene SLC5A1 coding for the sodium-glucose cotransporter 1 (SGLT1) was upregulated ($p < 0.01$) in Si50 compared to Co50, while the transport gene SLC16A1 was downregulated ($p < 0.01$) in Si50 compared to Co50. Furthermore, membrane surface associated genes such as MUC1 and MUC2 were upregulated ($p < 0.01$) in Co50 compared to Si50.

Expression of genes in mucosa of the small intestine was highly affected by sampling site (pre- vs post-PP), while treatment only affected MUC2 as pigs provided CON had greater expression of MUC2 than pigs provided TREAT during the suckling period ($p = 0.01$; Fig. 6). Genes coding for the following cytokines IL12B, IL10, IL1B were upregulated ($p = 0.01$), while IL1RAP was downregulated ($p = 0.02$) in mucosa sampled post-PP compared to pre-PP. In addition, the tight junction gene OCLN and the membrane surface associated gene MUC1 were upregulated in mucosa sampled pre-PP compared to post-PP ($p = 0.01$).

3.8. Short chain fatty acid content in digesta of E. coli inoculated pigs

Concentration of SCFA in digesta was not affected by milk formula treatment but was highly affected by segment of the gastrointestinal tract (Table 7). Acetic acid was detected in all segments and the concentration was greatest ($p \leq 0.04$) in caecum and Co50 compared to the stomach and Si50. In addition, the concentration of DL-lactic acid tended to be higher ($p = 0.09$) in the stomach than in Si50. Furthermore, the concentration of isobutyric acid, n-butyric acid, iso-valeric acid and n-valeric acid was lower ($p < 0.01$) in caecum than in Co50.

4. Discussion

During lactation, the Ca demands are greater than during gestation (Zeni et al., 1999). The Ca absorption in the intestine is mainly regulated by vitamin D (Lal et al., 1999). This might explain the decreased plasma concentration of 25-OH-D_3 observed in sows after farrowing, and the subsequent increase as lactation progressed. The concentration of various metabolites of vitamin D, including vitamin D_3, 25-OH-D_3 and 24(R),25-$(OH)_2$-D_3, was not detectable in most milk samples in the present study. Our results confirm previous research showing that sow milk contains a limited amount of vitamin D and its metabolites, and that sow milk as such is not a vitamin D vehicle for piglets (Matte and Audet, 2020). In the present study, milk formula was provided in addition to sow milk. Although the vitamin D supplementation to the milk formula had some influence on the vitamin D status of the piglets during suckling, the milk formula treatments were in general less effective in improving the vitamin D status of suckling piglets when compared to the use of UVB light in the study of Matte et al. (2017). Vitamin D provided to piglets in the form of 25-OH-D_3 may be a more efficient dietary source of vitamin D than vitamin D_3 to increase the vitamin D status in piglets during the suckling period. Thus, although the piglets' plasma concentration of 25-OH-D_3 in general was low when compared to the study by Madson et al. (2012) summarising reference plasma levels for pigs, our results seemed to indicate that provision of 25-OH-D_3 during the suckling period increased the vitamin D status in piglets at weaning compared to piglets provided vitamin D_3. Irrespectively of the minor difference in total vitamin D content of the milk formulas, our results indicated that 25-OH-D_3 is a more optimal vitamin D source to piglets than vitamin D_3 in agreement with other studies (Lauridsen et al., 2010; Konowalchuk et al., 2013; Weber et al., 2014).

Our results suggest that when pigs are provided 25-OH-D_3 rather than vitamin D_3 pre-weaning, more 25-OH-D_3 is available during the post-weaning period in which the immune system may be challenged. The present experiment furthermore showed that vitamin D is used during the activation of the immune system (Adams et al., 2009), as pigs inoculated with *E. coli* had lower plasma concentrations of 25-OH-D_3 than non-inoculated control pigs. The lower plasma concentration of 25-OH-D_3 in *E. coli* inoculated pigs compared to control pigs might be due to that the liver is mobilising vitamin D as a result of an *E. coli* infection, as also shown for the fat-soluble vitamin E (Lauridsen et al., 2011). Unfortunately, it was not possible to measure the bioactive form of vitamin D, i.e., 1α,25-dihydroxyvitamin D_3. However, when vitamin D is sufficient, the production of 1α,25-dihydroxyvitamin D_3 from 25-OH-D_3 is adequate and the excess of 25-OH-D_3 is converted to 24(R),25-$(OH)_2$-D_3 (Tang et al., 2019). Thus, it is expected that a high level of 24(R),25-$(OH)_2$-D_3 equals a high level of 1α,25-dihydroxyvitamin D_3.

A high vitamin D status per se indicates that more vitamin D is available in the body including the intestine, and might enhance the immune capacity of the intestine against *E. coli* infection, as for example by increasing the expression of antimicrobial peptides such as cathelicidin (Flohr et al., 2014). The reduced immunological responses of pigs provided 25-OH-D_3 as indicated by lower plasma concentrations of IgG and specific antibodies as compared to pigs provided vitamin D_3

Table 5
Haematological parameters as well as plasma concentrations of cytokines and CRP in *E. coli* inoculated pigs and control pigs on day 9 post-weaning provided CON or TREAT during the suckling period.[1]

Group	*E. coli* inoculated pigs		Control pigs			p-value		
Treatment	CON	TREAT	CON	TREAT	SEM[2]	Treatment	*E. coli*	Treatment × *E. coli*
WBC[3], g/L	16.0	15.9	14.8	15.5	1.51	0.81	0.76	0.85
Neutrophils, %	40.3	43.0	37.4	41.9	2.84	0.21	0.48	0.76
Lymphocytes, %	54.5	50.6	56.0	50.9	2.88	0.13	0.76	0.82
NLR[4]	0.78	0.91	0.70	0.85	0.10	0.17	0.49	0.95
Monocytes, %	4.80	5.64	5.92	6.15	0.41	0.21	0.047	0.51
Eosinophils, %	0.40	0.64	0.65	1.08	0.12	0.01	0.01	0.45
HGB[5], g/L	113	118	98.2	108	7.51	0.35	0.11	0.77
HCT[6], %	38.9	40.6	33.2	37.3	2.49	0.26	0.08	0.64
MCV[7], x10^{-15} L	56.0	59.6	54.1	56.7	2.71	0.26	0.38	0.84
MCH[8], pg/mL	16.2	17.2	16.0	16.4	0.78	0.37	0.48	0.70
MCHC[9], g/L	292	290	296	288	5.59	0.40	0.73	0.65
IFN-γ, ng/mL	11.4	18.9	39.6	25.7	4.01	0.10	0.01	0.03
IL-1α, ng/mL	0.041	0.017	0.014	0.031	0.02	0.44	0.99	0.65
IL-1β, ng/mL	0.38	0.18	0.08	0.32	0.12	0.73	0.59	0.31
IL-1RA, ng/mL	0.78	0.65	0.46	0.50	0.30	0.78	0.55	0.99
IL-4, ng/mL	0.67	0.23	0.06	0.52	0.20	0.50	0.93	0.15
IL-6, ng/mL	0.10	0.04	0.04	0.07	0.04	0.58	0.89	0.70
IL-8, ng/mL	0.04	0.06	0.06	0.04	0.01	0.18	0.92	0.18
IL-10, ng/mL	0.36	0.13	0.13	0.26	0.12	0.50	0.82	0.64
IL-12, ng/mL	0.80	0.85	0.83	0.92	0.13	0.59	0.67	0.92
IL-18, ng/mL	0.64	0.40	0.31	0.81	0.20	0.77	0.25	0.21
TNF-α, ng/mL	0.04	0.16	0.10	0.07	0.02	0.047	0.10	0.047
CRP, μg/mL	56.1	254	236	159	0.068	0.03	0.04	0.06

[1] Blood samples were obtained 9 days post-weaning (at day 36 of age). Values are LS means, n = 8.
[2] Average standard errors of LS means.
[3] WBC white blood cells.
[4] NLR neutrophils to lymphocytes ratio.
[5] HGB haemoglobin.
[6] HCT haematocrit.
[7] MCV the mean cell volume-the ratio of the haematocrit to the concentration of red blood cells.
[8] MCH the mean corpuscular haemoglobin-the ratio of the total mass of haemoglobin to the number of red blood cells.
[9] MCHC the mean corpuscular haemoglobin concentration – identifies the amount of haemoglobin in a single red blood cell.

Table 6
Concentration of IgA in intestinal mucosa of *E. coli* inoculated pigs provided CON or TREAT during the suckling period.

Treatment	CON		TREAT			p-value	
Sample site[1]	Pre-PP	Post-PP	Pre-PP	Post-PP	SEM[2]	Treatment	Sample site
IgA, μg /mL	40.1	79.5	53.0	90.5	10.3	0.43	< 0.001
Mucosal IgA, μg/mg protein mucosa	0.75	1.47	1.02	1.69	0.19	0.40	< 0.001

[1] Samples collected pre- and post-Peyer's patches (PP) in the small intestine, n = 8.
[2] Average standard errors of LS means.

during the suckling period might indicate an improved capability to clear the infection locally in the intestine upon the *E. coli* inoculation.

Our *E. coli* inoculation model was designed to exert a mild disease activity in vivo in the form of diarrhoea development, i.e., with no requirement for medical treatment. Sampling on day 9 post-weaning, i.e., 7 days post inoculation, would be expected to result in changes in the measured intestinal parameters, as day 5–6 post inoculation is considered the time by which the infection is at the highest level. In the present study, a greater faecal shedding of *E. coli* as well as a higher faecal concentration of enterotoxins on day 10 post-weaning was observed in pigs inoculated with *E. coli* compared to non-inoculated pigs indicating an increased growth of *Enterobacteriaceae* including *E. coli* and thus confirming a successful colonisation with *E. coli*. The obtained plasma measures on day 9 was only partly influenced by the *E. coli* challenge, and the infection seemed to be cleared in the intestine before challenging the system, i.e., no systemic impact was observed as the plasma CRP concentration in general was low and not increased on day 7 after the *E. coli* challenge. This does not preclude that systemic effects might have been discernible at an earlier time point after the *E. coli* challenge. In addition, the cytokine responses in plasma and haematological measures did in general not differ between inoculated and non-inoculated pigs; however, day 7 of sampling (after infection) is also close to the end of an acute phase for many biomarkers. The lack of impact of *E. coli* inoculation on haematological parameters may indicate that the infection was cleared locally and was not expanded to a state of systemic inflammation, which may also be explained by the enhanced specific antibody response against *E. coli*. It should be noted that during the pre-weaning period, piglets were also exposed to *E. coli* in the barn environment and via the sow faecal shedding, and this natural exposure may have initiated the development of an *E. coli* specific antibody response prior to *E. coli* inoculation. Interestingly, pigs provided 25-OH-D_3 as the vitamin D source seemed to be less affected by the *E. coli* infection as compared to pigs provided vitamin D_3, which may be due to an improved bioavailability of vitamin D for immune responses. Thus, piglets provided 25-OH-D_3 might be considered more immunologically robust against *E. coli* infection than pigs provided vitamin D_3 as the sole vitamin D source.

5. Conclusions

Provision of milk formula supplemented with 25-OH-D_3 to piglets while suckling the sow tended to improve the vitamin D status at weaning compared to piglets provided milk formula supplemented with vitamin D_3. An elevated vitamin D status at weaning might enhance the

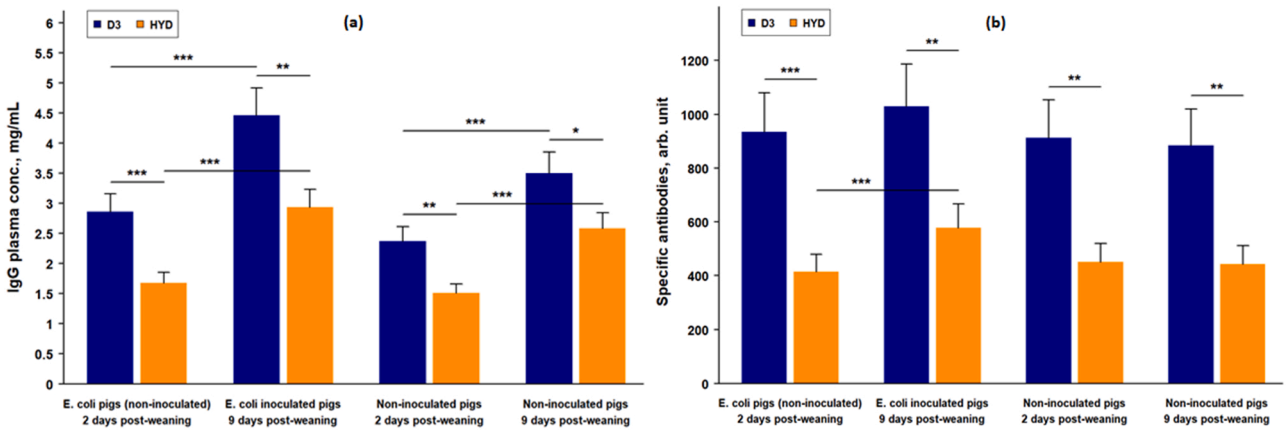

Fig. 4. IgG (a) and specific antibody (b) responses in plasma of *E. coli* inoculated pigs (before and after *E. coli* inoculation) and non-inoculated pigs on day 2 and 9 post-weaning allocated CON or TREAT during the suckling period. Values are LS means, and vertical bars represent standard errors, n = 8.

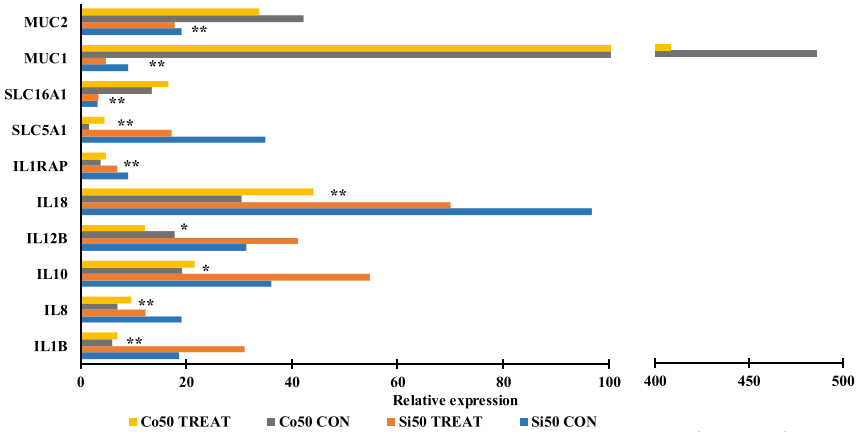

Fig. 5. Relative gene expression in different segments of the intestine of *E. coli* inoculated pigs provided CON or TREAT during the suckling period. Values are means, n = 8 for each experimental group. Significant differences within segments are indicated by stars (* < 0.05, ** < 0.01).

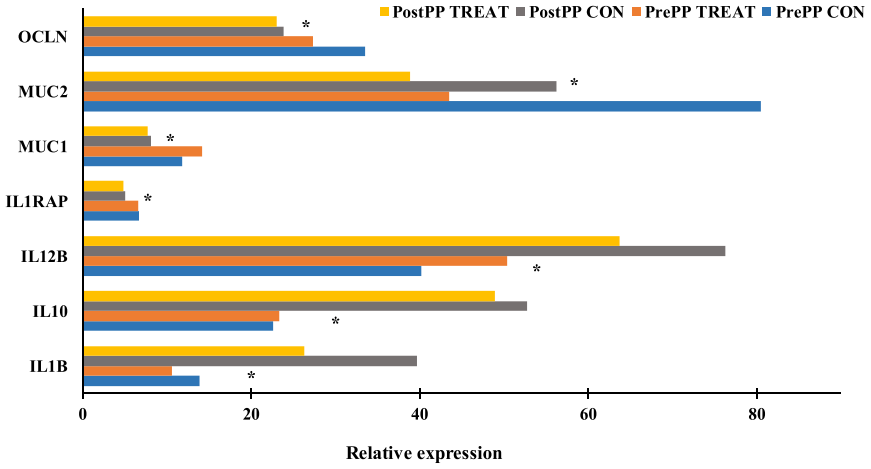

Fig. 6. Relative gene expression in mucosa pre- and post-Peyer's patches (PP) of *E. coli* inoculated pigs provided CON or TREAT during the suckling period. Values are means, n = 8 for each experimental group. Significant differences are indicated by stars (* < 0.05).

robustness of the intestine against *E. coli* infection. Furthermore, pigs provided 25-OH-D_3 as the vitamin D source might be more capable of resisting *E. coli* infection.

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Table 7
Concentration of SCFA (mmol/kg) in digesta from stomach, Si50, caecum and Co50 of *E. coli* inoculated pigs provided CON or TREAT during the suckling period.[1]

Segment	Stomach		Si50		Caecum		Co50		SEM[2]	p-value	
Treatment	CON	TREAT	CON	TREAT	CON	TREAT	CON	TREAT		Segment	Treatment
Formic acid	0.52	0.19	0.30	0.33	NA	NA	NA	NA	0.18	0.88	0.46
Acetic acid	4.04^{a}	3.12^{a}	2.31^{a}	1.17^{a}	43.7^{b}	42.6^{b}	46.0^{b}	46.8^{b}	6.00	0.04	0.86
Propionic acid	NA	NA	NA	NA	17.7	16.4	19.1	17.0	3.52	0.45	0.72
Isobutyric acid	NA	NA	NA	NA	0.55^{a}	0.33^{a}	1.76^{b}	1.43^{b}	0.18	< 0.01	0.11
n-butyric acid	NA	NA	NA	NA	3.94^{ac}	3.16^{ab}	6.60^{bc}	5.41^{c}	1.44	< 0.01	0.64
Iso-valeric acid	NA	NA	NA	NA	0.38^{a}	0.26^{a}	1.21^{b}	1.02^{b}	0.14	< 0.01	0.40
n-valeric acid	NA	NA	NA	NA	0.74^{a}	0.60^{a}	1.50^{b}	1.31^{b}	0.24	< 0.01	0.45
DL-lactic acid	16.5	15.5	10.1	5.9	NA	NA	NA	NA	4.42	0.09	0.57

[1] Mean values within a row with different superscript letters are significantly different, n = 8.
[2]Average standard errors of LS means.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2023.110557.

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