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Clostridioides difficile-mesocolonic oedema in neonatal suckling piglets develops regardless of the fibre composition in sow's diets

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ABSTRACT

Maternal dietary factors have been reported to influence *Clostridioides difficile* colonisation in the offspring. Twenty suckling piglets from sows fed diets supplemented with high-fermentable sugar beet pulp (**SBP**) or low-fermentable lignocellulose (**LNC**) fibres during gestation and lactation were dissected in the first week after birth. Postmortem analysis included clinical mesocolon and faecal scoring, concentration of *C. difficile* and respective toxins in colon digesta and faeces, immunoglobulins in serum and inflammatory markers in serum and colon tissues. Sow colostrum was assessed for nutrients, immunoglobulins and biogenic amines. Toxin-neutralising IgG antibodies were measured in colostrum and serum of the sows, and in colon digesta and serum of the piglets. Mesocolonic oedema of different severity was present in most of the piglets from both sows' feeding groups. Concentrations of *C. difficile,* toxins and calprotectin in colon digesta and faecal contents did not differ between the study piglets. Calprotectin correlated positively with mesocolon score (rho = 413, $P = 0.07$). Piglets from sows fed LNC vs SBP tended to have higher IgA ($P = 0.089$), IgG ($P = 0.053$), total Ig ($P = 0.053$), albumin ($P = 0.075$) and total protein content ($P = 0.007$) in serum. Colon tissues of piglets from the SFB vs LNC had upregulated expression of ZO-1 ($P = 0.021$), PCNA ($P = 0.015$) and TGF-β ($P = 0.014$). Titers of anti-toxin-IgG-antibodies in serum and colostrum and in piglet colon digesta and serum did not differ between sows from both dietary groups, but they all showed strong positive correlations. In conclusion, dietary sugar beet pulp or lignocellulose fed to sows did not influence the concentrations of *C. difficile* and toxins titers in colon digesta and faeces of neonatal piglets.

Implications

Clostridioides difficile-mesocolonic oedema in 4- to 6-day-old suckling piglets develops regardless of the fibre composition (either high-fermentable sugar beet pulp or low-fermentable lignocellulose) in sow's diets. Feeding sow's diets enriched in sugar beet pulp fibre as compared to lignocellulose fibre has a beneficial effect on intestinal barrier function, cell proliferation and immune response by means of upregulated gene expression of tight-junction protein zonula occludens 1, proliferating cell nuclear antigen and transforming growth factor-β in the colon tissues of their offspring. The mechanisms of protection from *Clostridioides difficile*-colon intoxication in neonatal piglets seem to be complex and call for more studies.

Introduction

Maternal factors during gestation and lactation have been reported to play a crucial role in the offspring development with long-lasting consequences on health in both animals and humans (Warner and Ozanne, 2010; Myles et al., 2013). Specifically, the nutritional status of gestating sows can have an impact on foetus and piglet physiology and development (De Quelen et al., 2010; Theil et al., 2014). After birth, neonatal piglets obtain necessary nutrients and bioactive compounds with colostrum and milk. Sow colostrum and faeces, besides the birth canal, are also an important source of microorganisms to a newborn piglet. This microbial inoculum is crucial for the microbial and immune programming and may define piglet health (Frese et al., 2015; Grześkowiak et al., 2019a). However, this sow-piglet association can be influenced by external factors such as maternal diet, which may modify microbiota, immune system and intestinal physiology (Awad et al., 2013; Paßlack et al., 2015). Thus, dietary strategies aiming

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at microbiota modulation towards the reduction of pathogen colonisation may contribute to a better resilience to environmental stress in a growing piglet.

Clostridioides difficile belongs to early microbial colonisers of the piglet's gut. *C. difficile* cell and toxin concentrations peak significantly during the first week of life, when the gut microbial diversity is still low (Grześkowiak et al., 2016; 2019a). During this time, piglets seem to be susceptible to *C. difficile* intoxication and can develop mesocolonic oedema (Songer and Uzal, 2005; Steele et al., 2010; Moono et al., 2015; Grześkowiak et al., 2016; 2017). In pigs, *C. difficile* infection (**CDI**) is localised mainly in the mesocolon, and no systemic infection has been reported so far. The main virulent factors associated with CDI in piglets and other animals and humans are *C. difficile* endotoxins, which are detrimental to intestinal epithelial cells (Nusrat et al., 2001; Grześkowiak et al., 2020). CDI in piglets is manifested by mesocolonic oedema, ascites, hydrothorax, typhlocolitis, often diarrhoea and fever; however, constipation has also been reported in CDI-affected piglets. Oedema in the mesocolon is due to disruption of epithelial integrity and infiltration of inflammatory mediators, which causes tissue damage. Imbalance of albumins between the plasma and tissues affects osmotic pressure leading to visible fluid accumulation in the mesocolon (Waters et al., 1998; Steele et al., 2010; Grześkowiak et al., 2017; 2020). The aetiology of mesocolonic oedema in neonatal piglets has been mainly attributed to *C. difficile* and its toxins. However, along with *C. difficile*, certain porcine viruses also have been identified in piglets affected with mesocolonic oedema (Derbyshire et al., 1975; Yaeger et al., 2002; Possatti et al., 2018). Whether mesocolonic oedema in neonatal piglets is caused mainly by *C. difficile* or whether certain viruses along with *C. difficile* are involved in its aetiology, requires further research. Indeed, studying CDI in suckling piglets faces many challenges. The models of CDI in naturally raised piglets are difficult to establish and do not seem to be reproducible (Lizer, 2010; Steele et al., 2010; Arruda et al., 2013; Grześkowiak et al., 2018). For yet not known reasons, some piglets may develop CDI, while others remain healthy. The predisposing factors to *C. difficile* colonisation and infection in piglets may involve an undeveloped gut microbiota or its dysbiosis, including functional alterations and possibly also insufficient anti-toxin antibody supply with colostrum among others (Blankenship-Paris et al., 1995; Arruda et al., 2013; Grześkowiak et al., 2019b). However, environmental factors such as diet also seem to play an important role in CDI development in neonatal piglets and hamsters (Blankenship-Paris et al., 1995; Grześkowiak et al., 2018). Noteworthy, the inclusion of certain dietary fibres in diets to gestating and/or lactating sows or as a creep feed to piglets have been associated with modulation of gut microbiota and immune parameters in suckling piglets (Correa-Matos et al., 2003; Werner et al., 2014; Paßlack et al., 2015). However, strategies aiming at prevention of *C. difficile* colonisation and infection development in suckling piglets have been poorly studied so far. We have previously shown that by manipulating the source of dietary fibres in sows' feeds during gestation and lactation, it is possible to influence the colonisation patterns of *C. difficile* in their suckling piglets (Grześkowiak et al., 2022). Therefore, the aim of this study was to explore whether the diets rich in either high- or low-fermentable fibres fed to sows during gestation and lactation have an impact on *C. difficile*-mesocolonic oedema development in their piglets.

Material and methods

Animals and sampling

A total of 20 healthy, average-BW piglets (German Landrace) housed with their littermates were euthanised between four and six days of life, which is when piglets are susceptible to *C. difficile* intoxication and can develop mesocolonic oedema (Songer and Uzal, 2005; Steele et al., 2010; Moono et al., 2015; Grześkowiak et al., 2016; 2017). The ratio of female and male euthanised piglets was 50/50 in each group. Each of the 20 piglets belonged to different mother sow.

The design of the feeding trial and the diet composition for sows have been described in detail in a previously published article (Grześkowiak et al., 2022). Briefly, the mother sows (n = 20) were fed isoenergetic and isonitrogenous diets which provided high inclusion percentage of high-fermentable fibre source in form of sugar beet pulp (**SBP**; inclusion rate: 15% sugar beet pulp, n = 10 sows) or high percentage inclusion of low-fermentable fibre source in form of lignocellulose (**LNC**; inclusion rate: 15% lignocellulose, n = 10 sows) during gestation and lactation. The farrowing occurred naturally without artificial induction. Lactation diets were provided to sows three days after farrowing. Water was available to the animals *ad libitum*.

The piglets were sedated with 20 mg/kg BW of ketamine hydrochloride Ursotamin®; Serumwerk Bernburg AG, Germany) and 2 mg/kg BW of azaperone (Stresnil®; Jansen-Cilag, Neuss, Germany). Thereafter, they were euthanised by intracardial injection of 10 mg/kg BW of tetracaine hydrochloride, mebezonium iodide and embutramide (T61®; Intervet, Unterschleißheim, Germany), as previously described (Grześkowiak et al., 2020).

Following sedation, blood was collected in tubes (S-Monovette® 7.5 ml, Clotting Activator/Serum, 92 × 15 mm, Sarstedt) and centrifuged at 2 000*g* for 10 min at room temperature to obtain serum. Following euthanasia, the gastrointestinal tract was removed, and tissue (1 cm^2) of the proximal colon was sampled, collected in RNA stabilisation reagent (RNAlater; Qiagen GmbH, Hilden, Germany), and kept for 2 h at +4 °C and then stored frozen at −80 °C until analysis. Colon digesta and rectal contents were collected and stored frozen at −30 °C until analysis. Findings of postmortem examination of the colon (to detect signs of mesocolonic oedema) were scored as follows: 1, normal; 2, mild oedema; 3, moderate oedema; 4, severe oedema. The seven-scale "Bristol stool form scale" was adapted to assess the faecal score for piglet samples (Lewis and Heaton, 1997). The faecal score was as follows: 1, separate and hard; 2, hard but lumpy; 3, soft with cracks; 4, soft and smooth; 5, soft blobs; 6, soft and mushy; 7, watery (diarrheic).

Colostrum (10 mL) was collected manually (without oxytocin injection) within 10 h after beginning of the farrowing and once after the placenta was expelled. Blood was collected from sows within 16 h postpartum by puncture of a jugular vein and transferred into S-Monovette Serum tubes with clotting activator (Sarstedt, Nümbrecht, Germany). Colostrum and serum samples were stored frozen at –20 °C for further analysis.

C. difficile, toxins and calprotectin in colon and faeces

Determination of *C. difficile* and toxins in colon digesta and faecal contents was determined on *C. difficile*-ChromID agar (Biomerieux, France) and using the ELISA commercial kit (tgcBIOMICS GmbH, Bingen, Germany), respectively. The methods have been previously described (Grześkowiak et al., 2016).

Calprotectin level in colon digesta and faecal contents was measured using the Porcine Calprotectin ELISA kit (MyBioSource, San Diego, CA) following the manufacturer's instructions.

Acute-phase proteins in piglet serum

C-reactive protein (**CRP**) and haptoglobin (**Hp**) concentrations were measured in serum using an automated bio-chemistry analyzer (Olympus AU600 Automatic Chemistry Analyzer, Olympus Europe GmbH, Germany) with commercial quantitative turbidimetric tests produced by SPINREACT, S.A.U (Spain) and Beckman

Coulter® (California, USA), respectively. Assays for CRP and Hp were performed as reported before (Hernández-Caravaca et al., 2017). Paraoxonase-1 (PON-1) was determined by spectrophotometric assay previously validated (Escribano et al., 2015) based on the rate of formation of p-nitrophenol at 405 nm using an automated chemistry analyzer (Olympus AU2700, Olympus Diagnostica GmbH, Hamburg, Germany). Adenosine deaminase (**ADA**) was analysed by a commercially available spectrophotometric automated assay (Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, CA, USA) previously validated in pigs (Contreras-Aguilar et al., 2020). Total protein and albumin were measured by commercially available kits produced by Beckman Coulter® (California, USA),

Gene expression in colon tissues

Total RNA from colon tissues (0.3 g) was extracted using NucleoSpin RNA Plus kit (Macherey–Nagel GmbH and Company KG, Düren, Germany) according to the manufacturer's instructions. The RNA quality and quantity were determined with the Agilent RNA 6000 Nano Kit (Agilent 2100 Bioanalyzer, Agilent Technologies, Waldbronn, Germany). Transcription into cDNA was performed using the SuperScript III Reverse Transcriptase First-Strand complementary DNA Synthesis System (Invitrogen) in a Sure Cycler 8800 (Agilent Technologies, Waldbronn, Germany). Quantitative real-time PCR (qPCR) was performed using the Brilliant II SYBR Green QPCR Master Mix with Low ROX (Agilent Technologies, Waldbronn, Germany) on a Stratagene MX3000p (Agilent Technologies, Waldbronn, Germany). The expression of the following genes was assessed: claudin 4 (**CLDN-4**), occludin (**OCLN**), zonula occludens 1 (**ZO-1**), mucin 1 (**MUC-1**), mucin 2 (**MUC-2**), mucin 5AC (**MUC-5AC**), mucin 20 (**MUC-20**), interferon-γ (IFN-γ), transforming growth-factor-β (**TGF-β**), tumour-necrosis-factor-α (**TNF-α**), interleukin-6 (**IL-6**), interleukin-8 (**IL-8**), interleukin-12 (**IL-12**) and proliferating cell nuclear antigen (**PCNA**). The succinate dehydrogenase subunit A (**SDHA**) and β2-microglobulin (**β2-glob**) were selected as housekeeping genes and used for data normalisation. Primer sequences and annealing temperatures are listed in Supplementary Table S1.

Analysis of nutrients in colostrum

As we previously described (Lugarà et al., 2022), crude fat and lactose were determined using standard procedures VDLUFA (VDLUFA VI 15.2.1, 20.2.3) ('Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) (2010) Methodenbuch Band VI.', 2010). Dumas nitrogen determination method was used to analyse the crude protein (Dumas, 1831). Urea and free amino acid concentrations were analysed using ion-exchange chromatography on a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd., Cambridge, UK). Briefly, 0.2 mL of each colostrum sample was treated with 0.05 mL of 10% sulfosalicylic acid. The samples were then mixed and stored at +4 °C for 30 min until complete precipitation. Thereafter, they were centrifuged, and 0.1 mL of the supernatant was mixed with 0.1 mL of lithium loading buffer (pH 2.2). The samples were separated (30 µL injection volume) on a lithium-ion-exchange column (Laborservive Onken GmbH, Gründau, Germany). Lithium buffer with different pH values was used as eluent. Urea was quantified after postcolumn ninhydrin derivatisation by photometric detection at 570 nm.

Biogenic amines in colostrum (putrescine, cadaverine, tyramine, histamine, spermidine and spermine) were analysed with ion-exchange chromatography as described previously (Pieper et al., 2014).

Immunoglobulins in serum of the piglets and sows and in colostrum

Immunoglobulins in serum and colostrum samples were assessed using ELISA commercial kits. The IgA and IgM were assessed using the Pig IgA ELISA kit and Pig IgM ELISA kit, respectively, following the manufacturer's instructions (Abcam, Cambridge, MA, USA). The IgG was analysed using a Pig IgG ELISA kit (Cat. No. E100-104, Bethyl Laboratories, Inc, Montgomery, TX, USA) with small alterations in the manufacturer's protocol. In brief, colostrum samples (100 µL each) were applied onto microtiter plate coated with goat anti-pig IgG antibodies (Bethyl Laboratories, Cat. No. A100-104A). After incubation, they were washed and subsequently treated with goat anti-pig IgG-HRP antibodies (Bethyl Laboratories, A100-104P), TMB (Sigma-Aldrich, T0440) and 2M H2SO4. The optical density was measured at the wavelength of 450 nm. A purified pig IgG (Bethyl Laboratories, Cat. No. P100-105) was used to construct a standard curve and to calculate the IgG concentration in the colostrum samples, as we previously described (Lugarà et al., 2022).

Anti-C. difficile-toxin-IgG antibodies in serum and colostrum of the sows and in serum and colon digesta of the piglets

The ELISA plates coated with either *C. difficile* toxin A (**TcdA**) or B (**TcdB**) proteins, diluent, wash buffer, TMB, stop reagent, specific mouse mABs against TcdA or TcdB as positive controls and mouse conjugate were purchased from (tgcBIOMICS GmbH, Bingen, Germany). The sera, colostrum and colon digesta samples were thawed at room temperature. They were gently homogenised by pipetting, diluted (1:100) in the sample diluent, and 100 µl of the diluted sample was added in duplicate to the plate wells. Wells with the sample diluent served as a negative control. The plates were then incubated at room temperature for 2 h and washed three times with the washing buffer. Thereafter, the 100 µl of conjugate goat anti-pig IgG-HRP (Bio-Rad AbD Serotec GmbH, Germany) at a dilution of 1:50 000 was added to the sera, colostrum, colon digesta and negative control wells, while 100 µl of mouse conjugate was added to positive control wells. The plates were then incubated at room temperature for one hour and washed three times. Then, 100 µl of TMB was added to each well and the reaction was stopped after 15 min with the addition of 50 µl of stop reagent. The measurement of the colour development was performed by a spectrophotometer at OD 450 nm.

Statistical analyses

The data were not normally distributed, and thus, they were analysed by Mann-Whitney U test. Fisher's Exact test was used to test the percentages of positive values for *C. difficile* and TcdB. Pearson chi-square test was used to test the faecal consistency and mesocolon scores. Correlation analyses were assessed by Spearman's correlation analysis procedure. Differences were considered significant at $P \leq 0.05$ (SPSS v. 27, Chicago, IL). The gene expression data of colon tissues were analysed by REST software (Qiagen GmbH, Munich, Germany) (Pfaffl et al., 2002). The ClustVis web tool was used for the Heatmap correlation plot using average clustering method (Metsalu and Vilo, 2015). The positive predictive value of mesocolonic oedema for *C. difficile* toxin was calculated by comparing the total number of toxin-positive piglets (colon digesta) with mesocolonic oedema to the total number of piglets with mesocolonic oedema.

Results

Gross observations

The average BW at the day of dissection was 2 033 g ± 115 for piglets from sows fed the diet supplemented with SBP and 2 112 g ± 96 for piglets from sows fed the diet supplemented with LNC ($P = 0.603$). Mesocolonic oedema of different severity was observed in dissected piglets from both dietary groups; however, there was no significant difference in the mesocolonic score between the two groups ($P = 0.601$) (Fig. 1a, c). While eight of 10 piglets from sows fed SBP developed any type of oedema (mild, moderate or severe), only six of 10 piglets from sows fed LNC showed similar findings ($P = 0.329$). There were no visible signs of other gastrointestinal infections in piglets. Faecal score (Fig. 1b) did not differ between the groups ($P = 0.655$), although faeces of piglets whose sows were fed diets high in LNC tended to have a softer faecal consistency than those of the sows fed SBP.

C. difficile and toxins in the colon digesta and faeces

C. difficile was detected in all analysed colon digesta and faecal samples. Concentrations of *C. difficile* in the colon digesta were numerically higher in piglets from sows fed SBP vs LNC ($\log_{10}$ 5.5 CFU/g vs $\log_{10}$ 4.9 CFU/g, $P = 0.696$) (Fig. 2a). In faeces, the level of *C. difficile* was higher in piglets from sows fed SBP vs LNC, however, without a significant difference between the study groups ($\log_{10}$ 5.8 CFU/g vs $\log_{10}$ 5.3 CFU/g, $P = 0.604$) (Fig. 2c).

In the colon digesta, 20% of the piglets from SBP-fed sows had detectable levels of TcdB, as compared to 50% of the piglets from the sows fed LNC ($P = 0.201$). In the faeces, TcdB was detected in 30% of the piglets from the sows fed SBP and 33% of piglets from the sows fed LNC ($P = 0.630$).

Concentration of TcdB in the colon digesta and in the faeces did not differ between the study groups ($P = 0.800$ and $P = 0.400$, respectively) (Fig. 2b, d).

Calprotectin in the colon digesta and faeces

Concentration of calprotectin in the colon digesta did not differ between the piglets from sows fed SBP or LNC ($\log_{10}$ 3.0 ng/g ± 0.01 vs $\log_{10}$ 3.0 ng/g ± 0.02, $P = 0.218$). Neither faecal calprotectin differed between the piglets from SBP- and LNC-fed sows ($\log_{10}$ 2.9 ng/g ± 0.01 vs $\log_{10}$ 2.9 ng/g ± 0.02, $P = 0.133$).

Immunoglobulins in piglet serum

There was a trend for increased concentrations of IgA ($P = 0.089$) and IgG ($P = 0.053$), but not for IgM ($P = 0.165$) in sera of piglets from sows fed LNC vs SBP. Total immunoglobulin content

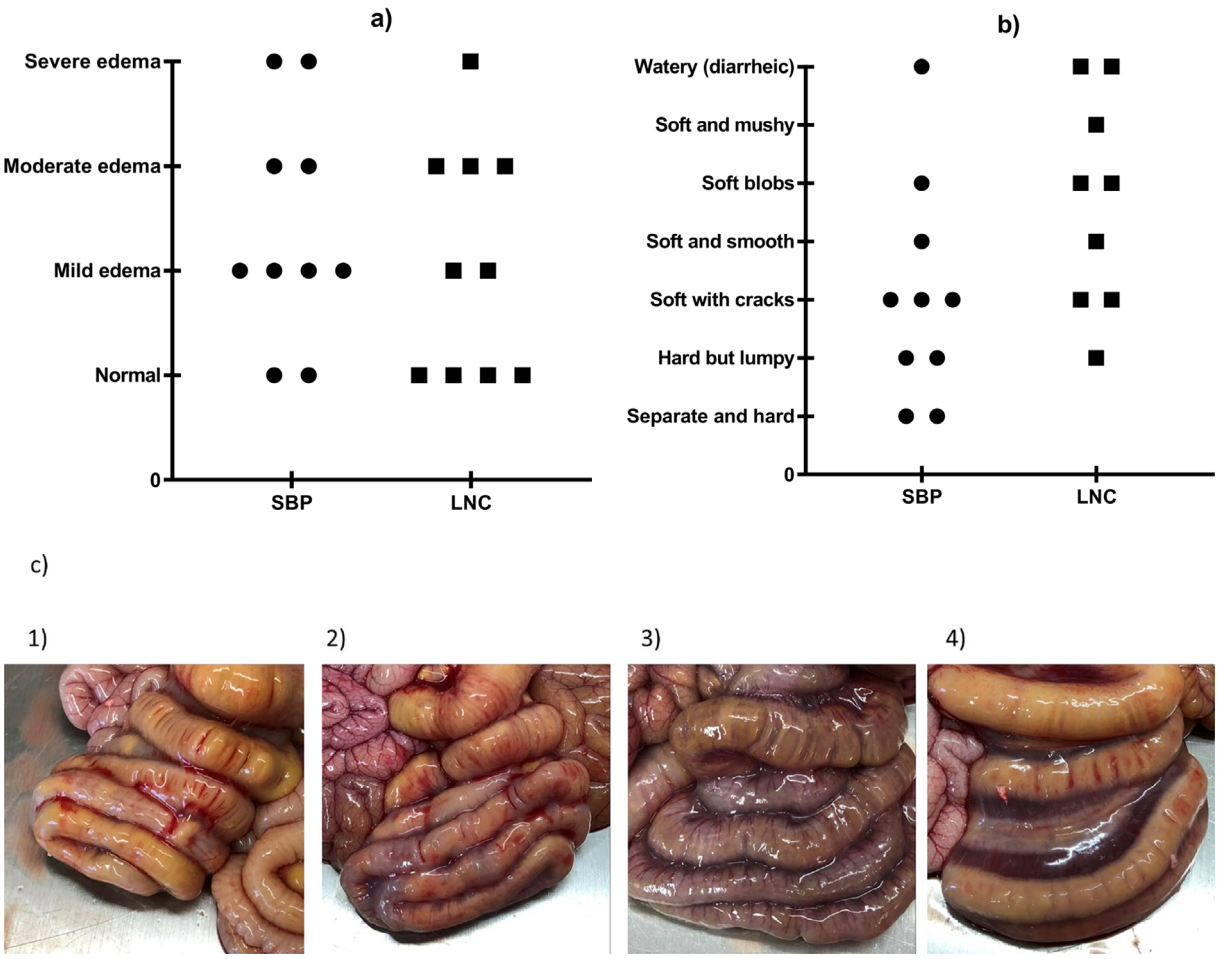

Fig. 1. Clinical mesocolon score (a) faecal score (b) and colon images (c) of the dissected piglets whose dams were fed diets containing high-fermentable sugar beet pulp (SBP) or low-fermentable lignocellulose (LNC) fibres during gestation and lactation. Colon image score (c): 1, normal; 2, mild oedema; 3, moderate oedema; 4, severe oedema. Faecal score: 1, separate and hard; 2, hard but lumpy; 3, soft with cracks; 4, soft and smooth; 5, soft blobs; 6, soft and mushy; 7, watery (diarrheic). Abbreviations, SBP = sugar beet pulp, LNC = lignocellulose.

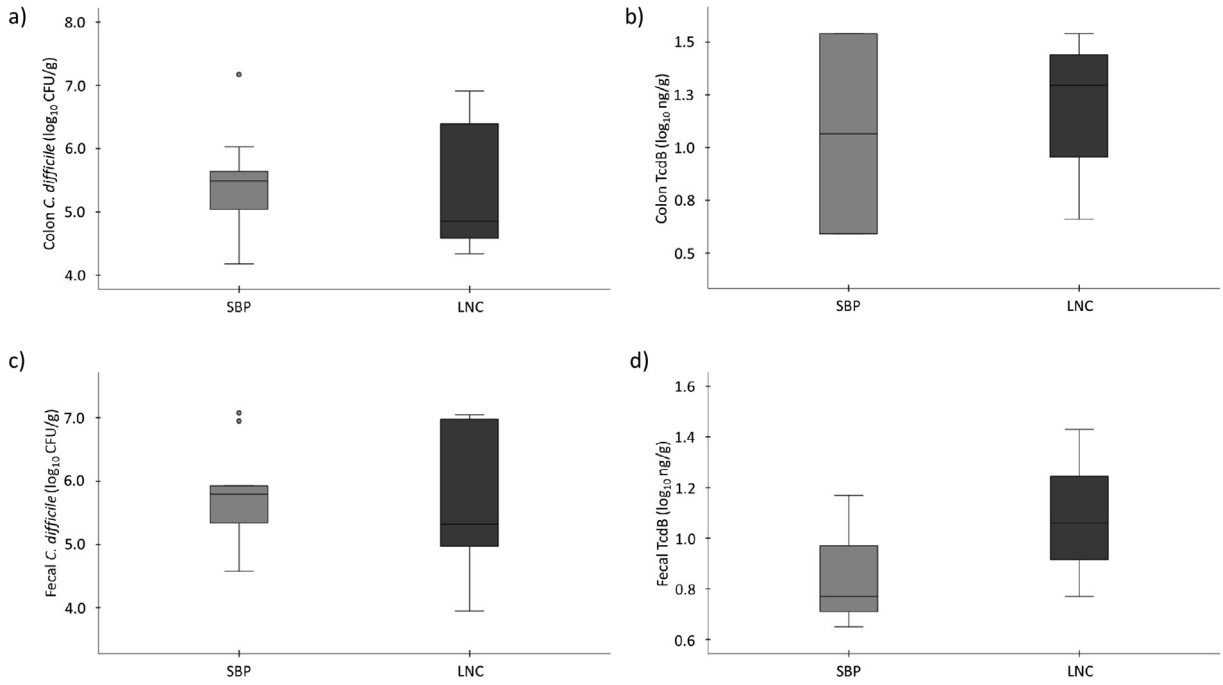

Fig. 2. Concentrations of *C. difficile* (log_{10} CFU/g) and toxin B (TcdB) (log_{10} ng/g) in the colon digesta (a, b) and faeces (c, d) of the dissected piglets whose dams were fed diets containing high-fermentable sugar beet pulp (SBP) or low-fermentable lignocellulose (LNC) fibres during gestation and lactation. Abbreviations: SBP = sugar beet pulp; LNC = lignocellulose; TcdB = toxin B Grey dots indicate outliers.

tended to be higher in piglets from sows fed LNC vs SBP ($P = 0.053$) (Table 1).

Acute-phase proteins in piglet serum

Total protein level in serum was significantly higher in piglets from sows fed LNC vs SBP ($P = 0.007$) (Table 1). There was also a trend for higher albumin concentration in piglets from sows fed LNC vs SBP ($P = 0.075$). On the other hand, serum C-reactive protein, haptoglobin, adenosine deaminase and paraoxonase-1 did not show significant differences between the animals ($P = 0.684$, $P = 0.853$, $P = 1.000$, $P = 0.481$, respectively).

Relative gene expression analysis in the colon tissues

Colon tissues of the piglets from sows fed diets enriched in SBP had upregulated the expression of ZO-1 gene by 1.7 times ($P = 0.021$), PCNA gene by 1.8 times ($P = 0.015$) and TGF-β gene by 3.2 times ($P = 0.014$), as compared to LNC (Fig. 3).

Table 1
Immunoglobulins and acute-phase proteins in serum of the dissected piglets whose dams were fed diets containing high-fermentable sugar beet pulp (SBP) or low-fermentable lignocellulose (LNC) fibres during gestation and lactation.

Item	SBP Mean ± SE	LNC	*P*-value
Immunoglobulins [mg/mL]			
IgA	2.3 ± 0.3	3.5 ± 0.7	0.089
IgG	38.7 ± 8.6	50.4 ± 7.9	0.053
IgM	0.8 ± 0.2	1.1 ± 0.2	0.165
Total Ig	41.8 ± 8.5	55.0 ± 8.2	0.053
Acute-phase proteins			
Albumin [g/dL]	1.4 ± 0.1	1.6 ± 0.1	0.075
C-reactive protein [mg/L]	2.1 ± 0.5	1.7 ± 0.3	0.684
Haptoglobin [g/L]	0.67 ± 0. 20	0.52 ± 0.18	0.853
Adenosine deaminase [U/L]	9.4 ± 0.7	10.0 ± 0.8	1.000
Paraoxonase-1 [U/mL]	3.8 ± 0.4	4.9 ± 1.1	0.481
Total proteins [g/L]	43.5 ± 1.0	51.7 ± 2.8	0.007

Abbreviations: SBP = sugar beet pulp; LNC = lignocellulose.

Nutrients and bioactive compounds in colostrum

The composition of colostrum from sows fed SBP and LNC is presented in Table 2. Crude protein content in colostrum showed a trend for a higher level from the sows fed SBP vs LNC diets ($P = 0.079$). Crude fat content in colostrum was not different between the dietary groups ($P = 0.408$). The concentration of lactose was significantly lower in colostrum from the sows fed SBP, as compared to LNC ($P = 0.031$). Urea and total free amino acid concentrations in colostrum were not different between the study sows ($P = 0.182$ and $P = 0.133$, respectively). Levels of crude protein and lactose showed a negative correlation (rho = −0.769, $P < 0.001$, for both study groups), (rho = −0.767, $P = 0.016$, for either SBP or LNC).

The concentration of immunoglobulins in colostrum did not differ between the sows fed SBP or LNC diets ($P = 0.436$ for IgA, $P = 0.436$ for IgG and $P = 0.133$ for IgM), or were differences detected in the concentration of total immunoglobulin content ($P = 0.165$). In colostrum collected from sows fed SBP diet, IgA consisted of 13.6%, IgG 85.0% and IgM 1.3% of the total immunoglobulins analysed. In colostrum obtained from sows fed LNC diet, IgA consisted of 12.6%, IgG 86.1% and IgM 1.3% of the total immunoglobulins analysed.

Putrescine, cadaverine, spermidine and spermine were present in colostrum from sows fed SBP diets, while histamine and tyramine concentrations fell below the detection limit. On the other hand, colostrum of the sows fed LNC feeds contained spermidine and spermine while putrescine, histamine, cadaverine and tyramine were not detected. The concentrations of single biogenic amines in colostrum did not differ between the sows fed SBP or LNC feeds ($P = 0.833$ for spermidine, $P = 0.247$ for spermine, $P = 1.000$ for tyramine, respectively). Total biogenic amines were significantly higher in colostrum from sows fed SBP than LNC ($P = 0.048$).

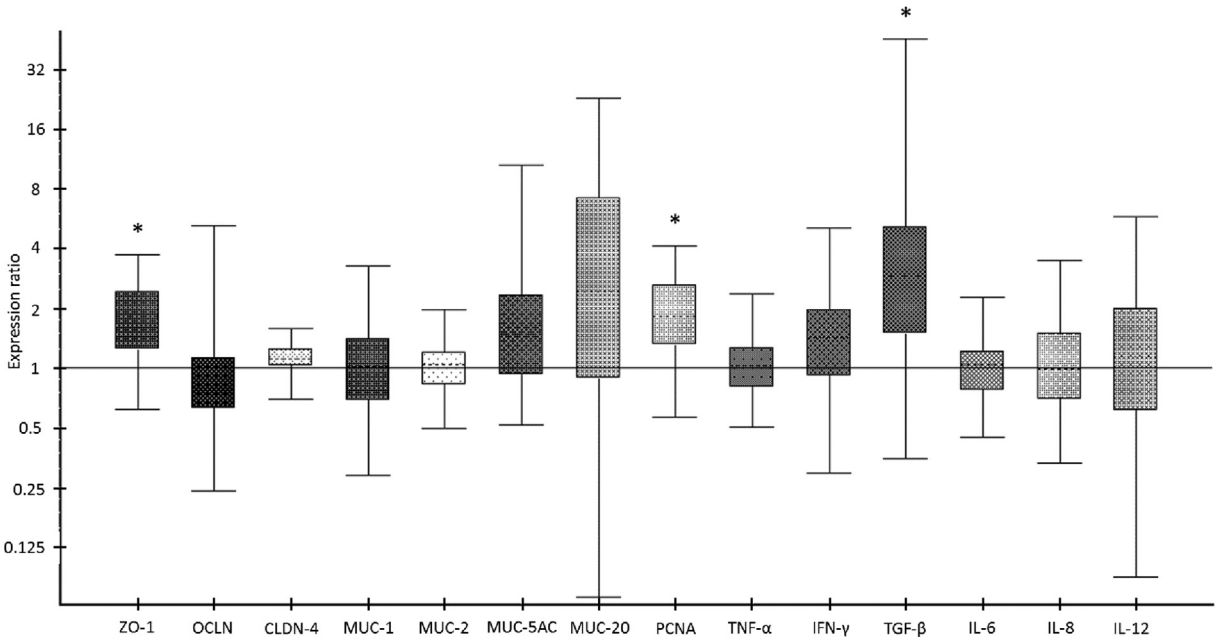

Fig. 3. Relative gene expression ratios (SBP/LNC) of tight-junction proteins, mucins, cell proliferation and immune markers in the colonic tissue extracted from the gut of piglets whose dams were fed diets containing high-fermentable sugar beet pulp (SBP) or low-fermentable lignocellulose (LNC) fibres during gestation and lactation. Abbreviations: SBP = sugar beet pulp; LNC = lignocellulose. Horizontal line on the y-axis denotes this same proportion of expression. Asterisk indicates significant difference ($P \leq 0.05$).

Table 2
Nutrients, urea, immunoglobulins and biogenic amines in colostrum (collected within 10 hours after farrowing) from the sows fed diets containing high-fermentable sugar beet pulp (SBP) or low-fermentable lignocellulose (LNC) fibres during gestation and lactation.

Item	SBP Mean ± SE	LNC	*P*-value
Nutrients and urea			
CP [g/kg]	194.0 ± 8.4	176.6 ± 4.7	0.079
Crude fat [g/kg]	38.8 ± 7.1	46.1 ± 6.0	0.408
Lactose [mg/g]	22.1 ± 1.2	25.6 ± 1.6	0.031
Urea [μmol/L]	1.9 ± 0.3	2.0 ± 0.37	0.182
Free amino acids[†] [μmol/g]	894.2 ± 176.6	624.3 ± 160.3	0.133
Immunoglobulins [mg/mL]			
IgA	20.4 ± 2.9	16.3 ± 1.3	0.436
IgG	127.9 ± 9.5	111.4 ± 8.8	0.436
IgM	2.0 ± 0.2	1.7 ± 0.2	0.315
Total Ig	150.4 ± 9.5	129.4 ± 8.9	0.165
Biogenic amines [μmol/g][*]			
Putrescine	1.7 ± 0.2	N/A	-
Histamine	N/A	N/A	-
Cadaverine	1.3 ± 0	N/A	-
Spermidine	9.3 ± 0.2	10.2 ± 1.2	0.833
Spermine	3.3 ± 0.5	2.5 ± 0.6	0.247
Tyramine	N/A	N/A	1.000
Total biogenic amines	13.8 ± 0.7	12.0 ± 1.7	0.048

Abbreviations: SBP = sugar beet pulp; LNC = lignocellulose.
N/A, not available (concentration below detection limit).
[†] Asp, Thr, Ser, Asn, Glu, Gln, Pro, Gly, Ala, Cit, Val, Cys, Met, Cys-Cys, Ile, Leu, Tyr, Phe, Orn, Lys.
[*] Number of samples (SBP/LNC) with detected biogenic amine: putrescine (3/0), cadaverine (1/0), spermidine (5/8), spermine (5/6), total biogenic amines (5/8).

Immunoglobulins in sow serum

Serum immunoglobulins (mean ± SE) were numerically higher in sows fed SBP vs LNC; however, they did not differ between the two groups: IgA (2.3 mg/mL ± 0.2 vs 2.3 mg/mL ± 0.3, $P = 0.631$), IgG (36.4 mg/mL ± 8.2 vs 31.9 mg/mL ± 8.5, $P = 0.631$), IgM (2.4 mg/mL ± 0.2 vs 2.2 mg/mL ± 0.3, $P = 0.353$), total immunoglobulins (41.0 mg/mL ± 8.1 vs 36.3 mg/mL ± 8.5, $P = 0.579$).

Anti-C. difficile-toxin-IgG in sera, colostrum and colon digesta

Serum and colostrum from sows fed SBP had numerically higher IgG-anti-TcdA-antibody titers, as compared to sows fed LNC ($P = 0.968$ for serum and $P = 0.968$ for colostrum). The same was noticed for serum and colostrum IgG-anti-TcdB-antibody titers in SBP- vs LNC-fed sows ($P = 0.968$ for serum and $P = 0.739$ for colostrum) (Table 3).

Colon digesta of piglets from sows fed SBP vs LNC had similar titers of IgG-anti-TcdA- and IgG-anti-TcdB-antibodies ($P = 0.383$ and $P = 0.536$, respectively). Neither serum IgG-anti-TcdA- nor IgG-anti-TcdB-antibody titers differed between piglets from sows fed SBP vs LNC ($P = 0.247$ and $P = 0.481$, respectively) (Table 3).

Independent of the dietary treatment, either IgG-anti-TcdA- or IgG-anti-TcdB-antibodies from sow's serum and colostrum and piglet's colon digesta and serum showed significantly ($P \leq 0.05$ and $P \leq 0.01$) strong positive correlations (Table 4).

Table 3
Anti-*C. difficile*-toxin A-IgG antibody (IgG-anti-TcdA) and anti-*C. difficile*-toxin B-IgG antibody (IgG-anti-TcdB) titers (OD_{450nm}) in colostrum and serum from the sows and in colon digesta and serum from their piglets. The sows were fed diets containing high-fermentable sugar beet pulp (SBP) or low-fermentable lignocellulose (LNC) fibres during gestation and lactation.

Item	SBP Mean ± SE	LNC	*P*-value
IgG-anti-TcdA			
Colostrum	0.97 ± 0.14	0.86 ± 0.11	0.968
Sow serum	0.53 ± 0.09	0.41 ± 0.03	0.968
Piglet colon digesta	0.09 ± 0.16	0.13 ± 0.34	0.383
Piglet serum	0.29 ± 0.43	0.38 ± 0.71	0.247
IgG-anti-TcdB			
Colostrum	0.61 ± 0.09	0.56 ± 0.12	0.739
Sow serum	0.26 ± 0.04	0.28 ± 0.04	0.968
Piglet colon digesta	0.07 ± 0.04	0.09 ± 0.13	0.536
Piglet serum	0.22 ± 0.31	0.29 ± 0.52	0.481

Abbreviations: SBP = sugar beet pulp; LNC = lignocellulose; IgG = immunoglobulin G; TcdA = toxin A; TcdB = toxin B.

Table 4
Spearman's rho correlation coefficient (and *P*-values) of either anti-*C. difficile*-toxin A-IgG antibody (IgG-anti-TcdA) or anti-*C. difficile*-toxin B-IgG antibody (IgG-anti-TcdB) titers ($OD_{450\ nm}$) from sow's serum and colostrum and piglet's colon digesta and serum, independent of the dietary treatment.

Item	Sow colostrum anti-TcdA-IgG	Sow colostrum anti-TcdB-IgG	Sow serum anti-TcdA-IgG	Sow serum anti-TcdB-IgG	Piglet colon contents anti-TcdA-IgG	Piglet colon contents anti-TcdB-IgG	Piglet serum anti-TcdA-IgG	Piglet serum anti-TcdB-IgG
Sow colostrum anti-TcdA-IgG	rho = 1.000							
Sow colostrum anti-TcdB-IgG	rho = 0.612 *P* = 0.005	rho = 1.000						
Sow serum anti-TcdA-IgG	rho = 0.707 *P* < 0.001	rho = 0.565 *P* = 0.012	rho = 1.000					
Sow serum anti-TcdB-IgG	rho = 0.732 *P* < 0.001	rho = 0.772 *P* < 0.001	rho = 0.740 *P* < 0.001	rho = 1.000				
Piglet colon contents anti-TcdA-IgG	rho = 0.162 *P* = 0.596	rho = −0.288 *P* = 0.318	rho = −0.058 *P* = 0.851	rho = 0.201 *P* = 0.511	rho = 1.000			
Piglet colon contents anti-TcdB-IgG	rho = −0.075 *P* = 0.799	rho = −0.345 *P* = 0.208	rho = −0.145 *P* = 0.620	rho = 0.044 *P* = 0.881	rho = 0.971 *P* < 0.001	rho = 1.000		
Piglet serum anti-TcdA-IgG	rho = 0.726 *P* < 0.001	rho = 0.436 *P* = 0.055	rho = 0.444 *P* = 0.057	rho = 0.537 *P* = 0.018	rho = 0.297 *P* = 0.302	rho = 0.111 *P* = 0.694	rho = 1.000	
Piglet serum anti-TcdB-IgG	rho = 0.679 *P* = 0.001	rho = 0.774 *P* < 0.001	rho = 0.700 *P* < 0.001	rho = 0.767 *P* < 0.001	rho = −0.136 *P* = 0.642	rho = −0.234 *P* = 0.401	rho = 0.756 *P* < 0.001	rho = 1.000

Abbreviations: SBP = sugar beet pulp; LNC = lignocellulose; IgG = immunoglobulin G; TcdA = toxin A; TcdB = toxin B.

Correlations, Heatmap associations and predictive value of mesocolonic oedema

Independent of the sow's dietary groups, positive significant correlations were found between *C. difficile* and TcdB in the colon digesta of piglets (rho = 0.606, *P* = 0.010). Mesocolonic oedema score correlated positively with colon digesta calprotectin, a non-specific marker of gut inflammation (rho = 0.544, *P* = 0.020). Piglet body weight did not correlate with mesocolonic oedema score (rho = −0.126, *P* = 0.596).

Heatmap associations demonstrated that IgG-anti-TcdA and -TcdB-antibody titers in colon digesta and serum of the piglets and immunoglobulins especially IgG in colostrum were positively associated with healthier mesocolon score in piglets from both dietary groups. *C. difficile*, toxins and calprotectin in either faeces or colon were positively associated with piglets with mesocolonic oedema. Older piglets (a range between 4 and 6 days of age) tended to show inflamed mesocolon. Moreover, colostrum IgA and IgG were positively associated with sows from SBP group (Fig. 4).

The positive predictive value of mesocolonic oedema for TcdB in either colon digesta or faeces was 31%.

Discussion

We have previously demonstrated that susceptibility to colonisation by gut pathogens, such as *C. difficile* in neonatal piglets, can be influenced by the sows' nutritional factors (Grześkowiak et al., 2022). Specifically, we showed that the sow's diets enriched with highly fermentable sugar beet pulp during gestation and lactation successfully reduced *C. difficile* shedding in suckling piglets compared to low-fermentable fibre lignocellulose. Our previous findings clearly support the phenomenon of the mother-offspring early microbial programming (Grześkowiak et al., 2022).

The current study further assessed whether these fibre-enriched diets fed to sows would have any impact on the mesocolonic oedema development in suckling piglets. Here, we show that the piglets developed mesocolonic oedema independent of the fibre type fed to their mothers. Specifically, we could demonstrate that mesocolon pathological- and faecal scores did not differ between the piglets whose mothers were fed diets enriched in sugar beet pulp or lignocellulose. Reports demonstrate that *C. difficile*-mesocolonic oedema develops spontaneously in neonatal suckling piglets and the reasons for that are not yet understood. Interestingly, studies demonstrate that not all piglets from the same litter are affected by mesocolonic oedema, indicating that specific factors may be involved in CDI development (Songer and Anderson, 2006; Squire et al., 2013). In addition, an involvement of other infectious agents such as certain types of viruses in mesocolonic oedema aetiology cannot be ignored and should be investigated in depth (Derbyshire et al., 1975; Yaeger et al., 2002).

In this study, *C. difficile* was detected in the colon digesta and faecal samples from all piglets, which indicates that this bacterium is present in the gut and is disseminated into the environment (Hopman et al., 2011; Grześkowiak et al., 2019a). On the contrary, toxin B was detected in the colon digesta of just 20% of piglets from the sugar beet pulp-fed sows and in 50% of piglets from the sows fed lignocellulose, while data from faecal samples showed no differences between the trial groups. This indicates that among *C. difficile*, there are also toxigenic ribotypes, which may possess the potential to intoxicate the gut and consequently lead to CDI development. Moreover, a lower prevalence of *C. difficile* toxins in piglets from sows fed sugar beet pulp is consistent with the findings from our recently published similar study in which piglets were screened for *C. difficile* toxins through the suckling period (Grześkowiak et al., 2022). However, in the present study, a higher number of replicates would be necessary to provide firm conclusions on the maternal fibre effect on mesocolonic oedema in their piglets.

The clinical mesocolon score varied from healthy to severe oedema in all the piglets. Other internal organs did not show gross-pathological signs. To date, there are no reports on the systemic CDI in piglets. Similarly, CDI in humans also includes local infection; however, a progressing severe toxic megacolon has been associated with fever and a rise in systemic inflammatory markers in patients with CDI (Rao et al., 2014). Here, we assessed the piglet colon digesta for calprotectin, a non-specific biomarker of gut inflammation in animals and humans (Burnham and Carroll, 2013; Hang et al., 2013). Although no differences were found in calprotectin concentration between the study piglets, this biomarker correlated positively with the mesocolon clinical score, indicating an increased local inflammation process in piglets who suffered from more severe mesocolonic oedema. Similarly, positive correlations between colon or faecal calprotectin and CDI-associated

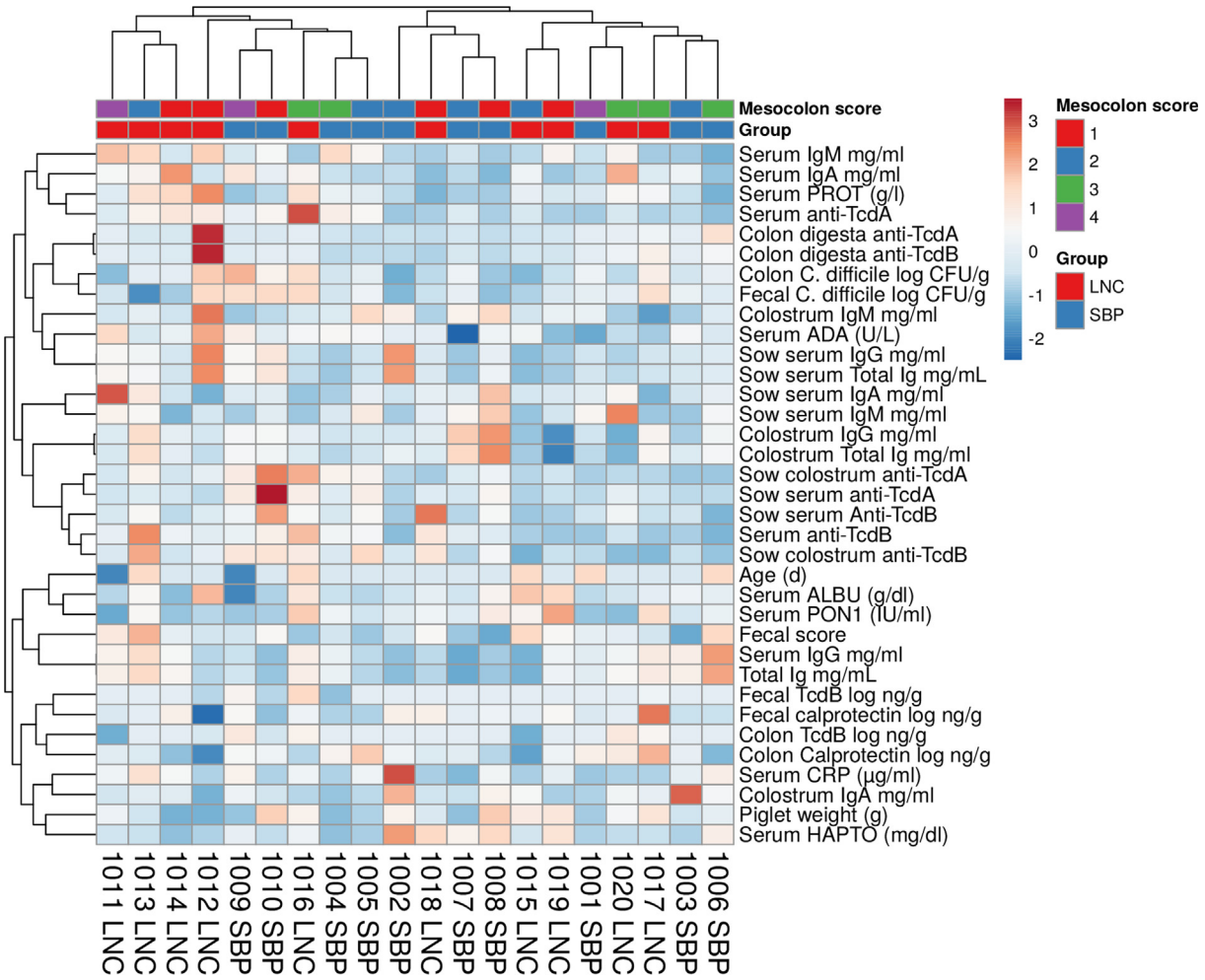

Fig. 4. Heatmap correlation plot of the certain measurements analysed in the piglets and sows (serum and colostrum only). Abbreviations: SBP = sugar beet pulp-enriched diet; LNC = lignocellulose-enriched diet.

parameters e.g. *C. difficile*, toxins or mesocolon score have been reported previously in piglets and humans (Peretz et al., 2016; Grześkowiak et al., 2018).

Interestingly, feeding the sows diets enriched in sugar beet pulp as compared to lignocellulose upregulated the gene expression of ZO-1, PCNA and TGF-β in colon tissues of the piglets. ZO-1 proteins are involved in signal transduction at the cell-to-cell level and may anchor other TJ-proteins such as occludin and cytoskeletal F-actin. Its expression may contribute to the strengthening of the intestinal barrier against gut pathogens and other harmful antigens (Bhat et al., 2020; Grześkowiak et al., 2020). Previous studies report on an increased gene expression of ZO-1 in colon tissue of weaned piglets fed soluble fibre (Chen et al., 2019). An increased expression of PCNA in colon tissue of piglets from the sugar beet pulp group may indicate a higher cell turnover but also a better protection of the host DNA during cell proliferation (Huygelen et al., 2014; González-Magaña and Blanco, 2020). We also noted a higher expression of TGF-β in piglets from sows fed sugar beet pulp vs lignocellulose group. It has been previously reported that a maternal soluble fibre diet increased the plasma concentrations of TGF-β in piglets (Cheng et al., 2018). Therefore, in the present study, maternal diet supplementation with sugar beet pulp had a positive influence on the intestinal barrier function, immune regulation and mechanisms of DNA synthesis and cell proliferation. The observed alterations may result from a different impact of sugar beet pulp- and lignocellulose-enriched diets on microbial composition and activity in the sows and in their offspring, as we have recently reported (Grześkowiak et al., 2022).

To assess whether the presence of mesocolonic oedema in piglets causes systemic inflammation, serum from the dissected animals was assessed for relevant biomarkers, which are associated with systemic inflammation in pigs (Pallarés et al., 2008; Escribano et al., 2015). We found that the health status of the mesocolon in piglets was not associated with statistically significant differences in the serum level of acute-phase proteins in the piglets. However, the elevated albumins and total protein content were significantly higher in serum of the piglets from sows fed lignocellulose vs sugar beet pulp, indicating that sows from the first group might have favoured their transfer with colostrum to their offspring. Noteworthy, some of the analysed acute-phase proteins such as haptoglobin or albumin can be transferred to suckling piglets with colostrum or milk indicating that a sow can exert systemic effect on the immune responses in their offspring (Carlsson et al., 1977; Hiss-Pesch et al., 2011). Other inflammatory markers in serum such as C-reactive protein, haptoglobin, adenosine deaminase and paraoxonase-1 did not differ between the study piglets.

It is intriguing that piglets with severe mesocolonic oedema do not show other symptoms of infection such as fever, diarrhoea, constipation or anorexia among others (Kyne et al., 2000; Grześkowiak et al., 2018). These asymptomatic piglets often over-

come the infection, since mesocolon of piglets dissected at the age of three or more weeks usually shows no signs of oedema, as is constantly observed during dissections in our animal facilities. Several factors may be responsible for a time-dependent protection from severe CDI. Among them, individual microbiota composition and activity, or efficient uptake of nutrients and bioactive compounds from colostrum, could to some extent protect from infection progress into more severe state (Quesnel, 2011; Theil et al., 2014; Grześkowiak et al., 2019b; 2020). For instance, piglets who are born first and with a higher body weight than their other littermates may have better access to teats to suckle colostrum. This could potentially have an impact on the efficient uptake of protective antibodies and improve piglet survival (Cabrera et al., 2012; Le Dividich et al., 2017). In our study, we chose piglets for dissections with the average body weight, thus, we did not observe any clear link of mesocolonic oedema with the piglet body weight, as assessed by correlation and Heatmap analyses.

To better understand determinants of protection, colostrum of the study sows was assessed for nutrients, immunoglobulins and biogenic amines. Indeed, the modulation of colostrum composition by nutritional factors has important implications on the offspring's health (Vallet et al., 2013; Loisel et al., 2013; Krogh et al., 2015). In humans, relationships between maternal diet and colostrum or breastmilk are not consistently reported, and available results are diverse. Specifically, the composition of fatty acids, proteins, vitamins and minerals but not carbohydrates in breastmilk has been reported to be dependent on maternal nutrition, as previously reviewed (Bravi et al., 2016). In sows, it has been shown that among different fibres, the inclusion of sugar beet pulp in the diet can influence the physiology and colostrum composition, with a positive output on piglets' well-being (Loisel et al., 2013; Krogh et al., 2015). Here, lactose content in colostrum was significantly higher in sows fed lignocellulose, while protein level showed a trend to be lower in this feeding group. Lactose and protein in colostrum are produced by the mammary glands during the lactogenesis (Neville and Morton, 2001; Devillers et al., 2004). It is possible that the two fibre diets differentially affected the closure of epithelial tight junctions, which resulted in observed differences in nutrient content in colostrum; however, these findings should be further explored. In addition, we observed a negative correlation between protein and lactose content in all colostrum samples, also depending on the feeding group. Such observations have also been noted before and may be related to the fact that a sow prioritises proteins over lactose in colostrum as an essential source of immunoglobulins and immune cells for their newborn piglets (Hurley, 2015; Craig et al., 2019). Contrary to humans, due to specific anatomy of porcine placenta, piglets are born immune-naïve and need to acquire essential antibodies and immunoglobulins with colostrum (Stokes, 2017). The immunoglobulin content in the colostrum did not differ significantly between sows fed sugar beet pulp or lignocellulose diets. It is however possible that other specific bioactive colostral compounds such as oligosaccharides, growth hormones, immune cells and microbes among others may also have affected the gut microbiota development in piglets (Zhang et al., 2018) with a consequence on *C. difficile* colonisation, as also observed in our previous study (Grześkowiak et al., 2022).

We have also determined immunoglobulins in serum and IgG antibodies against *C. difficile* toxins A and B in sow serum and colostrum. In addition, we have also detected IgG antibodies against *C. difficile* toxins A and B in piglet colon digesta and serum. Although there were no statistical differences in antibody titers in either sera, colostrum or colon digesta from the animals of the two fibre groups, we could demonstrate strong significant correlations between IgG antibodies against *C. difficile* toxins A or B from sows and piglets. The maternal IgG antibodies against *C. difficile* toxins A and B are especially important for the passive protection of piglets against certain antigens such as *C. difficile* toxins. Previously, we showed that sow serum and colostrum contain anti-*C. difficile*-toxin-IgG antibodies and sow colostrum was able to protect the porcine intestinal cell lines IPEC-J2 and colon epithelium from toxin-detrimental effects in a time-dependent manner (Grześkowiak et al., 2019b; 2020). Animal studies demonstrate that the administration of human monoclonal anti-toxin-IgG-antibodies protects from CDI development and minimises gastrointestinal lesions in gnotobiotic neonatal piglets (Steele et al., 2013; Cohen et al., 2014). However, in naturally reared piglets as in the present study, the scenario is different and other factors may be involved in susceptibility and protection from CDI development. Additionally, since the immune system is still developing in neonates, piglets can acquire protective anti-toxin-antibodies with colostrum only. On the contrary, in adult humans, no evidence has been found of immune protection against colonisation by *C. difficile* and infection development by means of own serum levels of anti-toxin-A-IgG-antibodies (Kyne et al., 2000). However, the potential of intravenously administered toxin-neutralising antibodies has been observed in patients suffering from recurrent CDI (Alonso and Mahoney, 2019).

Moreover, we have also characterised biogenic amines in porcine colostrum since they have been reported in porcine colostrum and milk (Cheng et al., 2006). Although single biogenic amines did not differ between the colostrum from sows fed sugar beet pulp and lignocellulose, the concentration of total biogenic amines was slightly higher in the first dietary group. Previous animal studies demonstrated the beneficial impact of polyamines on intestinal cell proliferation, immune system and gut microbial communities when mice were fed human formula supplemented with biogenic amines (2.10–8.40 μg of putrescine, 22.05–88.20 μg of spermidine and 38.00–152.00 μg of spermine), as compared to human formula alone (Gómez-Gallego et al., 2017). The action of polyamines is mainly via marked effects on the structure and function of genomic DNA molecules, and they have been shown to be essential for the growth and development of the neonatal small intestine (Manjarin et al., 2014). In suckling piglets, oral administration of polyamines or proline has been shown to improve growth performance likely due to increased intestinal absorption, improved maturation of the intestinal mucosa and thus an improvement in epithelial restitution and barrier function after stress induction (Wang et al., 2015; van Wettere et al., 2016). Therefore, the presence of biogenic amines in colostrum and their role on piglet's health cannot be overruled and needs to be further explored.

C. difficile toxins can be easily determined from faeces, but a high concentration of toxins found in excreta does not necessarily correlate with mesocolonic oedema severity in neonatal piglets, as we observed here. However, we showed that the concentration and prevalence of toxins correlated positively between the colon digesta and faeces indicating that faeces may be a useful sample type to reflect the toxin concentration in the colon digesta of piglets. Interestingly, none of the piglets with a healthy mesocolon had detectable levels of toxins suggesting an involvement of toxins in mesocolonic oedema, as already mentioned. However, the positive predictive value of mesocolonic oedema for toxins in the colon digesta and faeces was only 31%, indicating that the determination of toxins in either the colon digesta or faeces as a sole parameter can be hardly linked to mesocolonic oedema. Thus, clinical examination of the mesocolon is necessary to confirm the presence/absence of oedema in piglets so far. Indeed, since mesocolonic oedema occurs spontaneously in neonatal piglets and not all piglets are evenly affected by it, even a much larger sample size may not be sufficient to draw firm conclusions. Interestingly, in humans, microbiome data were proposed to discriminate patients with CDI from those with non-CDI-associated diarrhoea and healthy controls (Schubert et al., 2014). Similarly, a development

of specific microbiological or inflammatory markers in faeces or blood with a combination of existing ones would be necessary to estimate the severity of mesocolonic oedema without a need to dissect a piglet.

Conclusions

Taken together, dietary sugar beet pulp or lignocellulose fed to sows did not influence the concentrations of *C. difficile* and toxins titers in colon digesta and faeces of neonatal piglets. Newborn offspring of the study sows seem to be equally prone to *C. difficile*-mesocolonic oedema. Whether bioactive compounds including specific anti-toxin-IgG-antibodies in colostrum may provide a first line of defence against infection progress in the mesocolon of neonatal piglets needs further investigation. The mechanisms of protection from *C. difficile*-colon intoxication in neonatal piglets seem to be complex and call for more studies. Dietary strategies in sows to control *C. difficile*-mesocolonic oedema in piglets are needed.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2022.100697.

Ethics approval

The institutional and national guidelines for the care and use of animals were followed, and the study was approved by the State Office of Health and Social Affairs 'Landesamt für Gesundheit und Soziales Berlin' (LAGeSo Reg. G0112/19). The ethical approval included the use of 20 sows in the feeding trial in regard to the power of the study. Dissection of one neonatal piglet per sow was approved in regard to well-being of the sows and the litters. This study was conducted in the experimental pig facilities of the Institute of Animal Nutrition at the Freie Universität Berlin in Berlin in Germany, as previously described (Grześkowiak et al., 2022).

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available to reviewers, or available from the authors upon request.

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Author contributions

Ł. G., W. V. and **J. Z.** designed the research study. **Ł. G., E-M. S., B. M.-V., A. G. W.** and **K. M.** conducted the animal trial. **J. J. C.** performed analyses of acute-phase proteins. **Ł. G.** analysed and interpreted the data and drafted the manuscript. **Ł. G., W. V.** and **J. Z.** had primary responsibility for the final content. All authors reviewed and approved the final manuscript.

Declaration of interest

The authors declare to have no conflicts of interest.

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