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Neonatal and maternal dietary interventions driving microbiota and functionality in piglet gut compartments

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Feed additives aiming to improve gastrointestinal health are frequently supplied to piglets after weaning (d28) but might be more effective when administered before weaning. In this period, feed additives can either be administered directly to neonates, or indirectly via sow's feed. It is yet unknown what the effect of the administration route is on gut functionality and health in the piglets. Therefore, we compared the effect of different dietary interventions on gut functionality after maternal administration (lactation feed) to the neonatal administration route (oral gavage). These feed interventions included medium chain fatty acids (MCFA), beta-glucans (BG), and galacto-oligosaccharides (GOS). For the maternal administration route, MCFA showed a significant difference in alpha diversity parameter, observed species at d1 and one differentially expressed gene (DEG), and 99 DEG at d31. Pathway enrichment analysis showed association to immune processes and metabolism. For BG, only 21 DEG were observed at d31, these DEGs were associated to signal transduction and sympathetic nerve pathway. For GOS, 816 DEG were observed for GOS at d1, and 77 at d31, where DEGs at d1 were associated to immune processes. For the neonatal administration route, MCFA showed 94 DEG and GOS 6 DEG. Where DEGs in MCFA were mainly associated to cell adhesion processes. When comparing the administration routes directly between treatment groups, we observed significant differences in alpha diversity parameters, observed species at d31 for MCFA, Shannon for GOS, as well as for beta diversity in GOS. For MCFA 515 DEG were observed, for BG 503 DEG, and for GOS 996 DEG. Where for MCFA most pathways were associated to immunological processes, BG showed more metabolism, and GOS mainly metabolism with a few immunological processes. The type of intervention and the administration route influence gut functionality of the piglets. MCFA administration led to a more differentially orchestrated response when comparing the neonatal and maternal administration route then the other two additives. This implies that for each nutritional intervention in early life of a pig the optimal route of administration needs to be determined.

Keywords Gut functionality, Gut development, Pigs, Dietary intervention, Microbiota, Intestinal gene expression

Abbreviations

BG	beta-glucan
DEG	Differentially expressed gene
ELISA	enzyme-linked immunosorbent assay
FDR	false discovery rate
HRP	horseradish peroxidase
Ig	Immunoglobulin
MCFA	medium chain fatty acid
TMB	3,3′,5,5′-Tetramethylbenzidine

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The gastrointestinal tract of pigs performs many functions beyond nutrient absorption. It acts as a physical barrier, engages in immune defense, detoxification, and lipid and protein metabolism. Gut functionality is affected by the intestinal microbiota, which, in turn, influences the performance, metabolism, and host gene expression[1–4]. In addition, microbiota composition of pigs is linked to maturation and functioning of the immune system and is therefore critical for overall health[5–9]. The environment including the maternal influence plays a key role in colonization of the gastrointestinal tract, and the period directly after birth is most critical for its colonization[10,11]. Interventions and changes in this early developmental period could therefore have long-term health effects by shifting the microbiota composition and thereby optimizing intestinal functionality.

Promising interventions include feed additives that improve gut functionality. A wide variety of additives are available, including pro- and prebiotics. These additives impact gut development in several ways, although for many additives mechanistic details are still lacking. The efficacy of feed additives is linked to several factors, such as intervention route and time of intervention. Until now, the impact of additives has mainly been investigated in weaned pigs when these additives can be administered via solid feed. However, administering interventions before weaning might be more effective to optimize growth and increase resilience around weaning and later in life[12]. This could reduce the use of antibiotics that are still frequently required to treat infections that are prevalent around weaning, such as post weaning diarrhea[13]. Moreover, the period before weaning appears to be a window-of-opportunity for microbial colonization and preweaning supplementation might therefore have longer-lasting effects compared to postweaning administration of feed additives[14].

When dietary interventions are supplied before weaning, two administration routes are possible: indirectly via the sow's lactation feed, or directly to piglets e.g., via oral gavage. After neonatal administration, additives reach the intestine of the piglet unmodified and have an immediate effect. Maternal administration can affect gut health of piglets in several ways: via sows' milk, vaginal microbiota, or via contact with sow's feces. In addition, *in utero* development can be influenced when interventions are supplied early in gestation. Maternal administration is less laborious compared to neonatal administration by oral gavage, and therefore easier to implement in practice. Yet it is unknown what the efficacy is of a dietary intervention administered to sows. Therefore, we examined the effect of three different dietary interventions supplied via both maternal or neonatal administration routes on gut functionality by comparing the impact of those administration routes on microbiota and gene expression of piglets.

These three feed additives were selected based on their different mode-of-action and comprised (1) medium-chain fatty acids (MCFA), (2) β-glucans (BG), and (3) galacto-oligosaccharides (GOS). The rationale for selecting medium chain fatty acids (MCFA), beta-glucans (BG), and galacto-oligosaccharides (GOS) as the primary feed additives for this study is based on their distinct modes of action and proven effects in the target animal, with these interventions chosen as showcase examples. MCFAs are naturally present in sows' milk and are absorbed in the upper jejunum where they exert antimicrobial and immunomodulatory activity[15]. BG are carbohydrates present in the cell walls of several yeasts, fungi and cereals and have immunomodulatory properties that affect both innate and adaptive immunity. The effect of β-glucans is largest in the ileum[16,17], in addition, in mice evidence has been presented that the gut microbiota and its metabolites were modulated as well as reduction of neuroinflammation[18]. Additionally, in pigs also several studies have also shown an effect of BG on immune related processes[19,20].

GOS are present at low concentrations in sows' milk as well, and there is evidence that they reach the colon (relatively) intact[21]. GOS serve as substrate for microbial species that are considered beneficial to the host and thus act as prebiotic[22,23].

The effect of maternal and neonatal administration of these additives on gut functionality was investigated by looking at the microbiota diversity and composition and local host gene expression in the specific gut segments, i.e., jejunum for MCFA, ileum for BG, and colon for GOS. Changes observed in the microbiota diversity and composition and intestinal gene expression can be related to gut health, while changes in the host intestinal gene expression also reflects the biological activity of the respective feed additives[24,25]. Our results reveal that the intestinal development can be modulated by different feed additives, leading to changes in microbiota composition and in local intestinal gene expressions. Moreover, the effect on intestinal development depends on the feed additive and the administration route.

Results

Performance

No significant differences in the growth performance, i.e. body weight, of piglets were observed between feed additive and control groups for both the maternal and neonatal administration route (Table S2). Do note that this study was not designed to find such differences.

The effect of maternal dietary interventions on Sow milk Immunoglobulins, and vaginal microbiota

Maternal dietary interventions can affect gut health of piglets either directly or indirectly. One option is that administration of the diet to sows leads to a difference in immunoglobulins in milk. Therefore, we first assessed whether the consumption of MCFA, BG, and GOS influenced the concentration of immunoglobulin (Ig) in colostrum and milk. We focused on total Ig, IgA, and IgG (Fig. 1). No significant differences of total Ig and IgG were measured between samples (colostrum and milk) of sows fed with different dietary interventions ($P > 0.10$).

Next to milk composition, the vaginal microbiota indirectly impacts gut colonization during farrowing. In addition, the vaginal sow microbiota could also impact gut colonization of the offspring, as piglets will encounter this microbiota during birth. No significant differences in alpha and beta diversity in vaginal swabs were observed between sows fed with the different dietary interventions (Fig. S1).

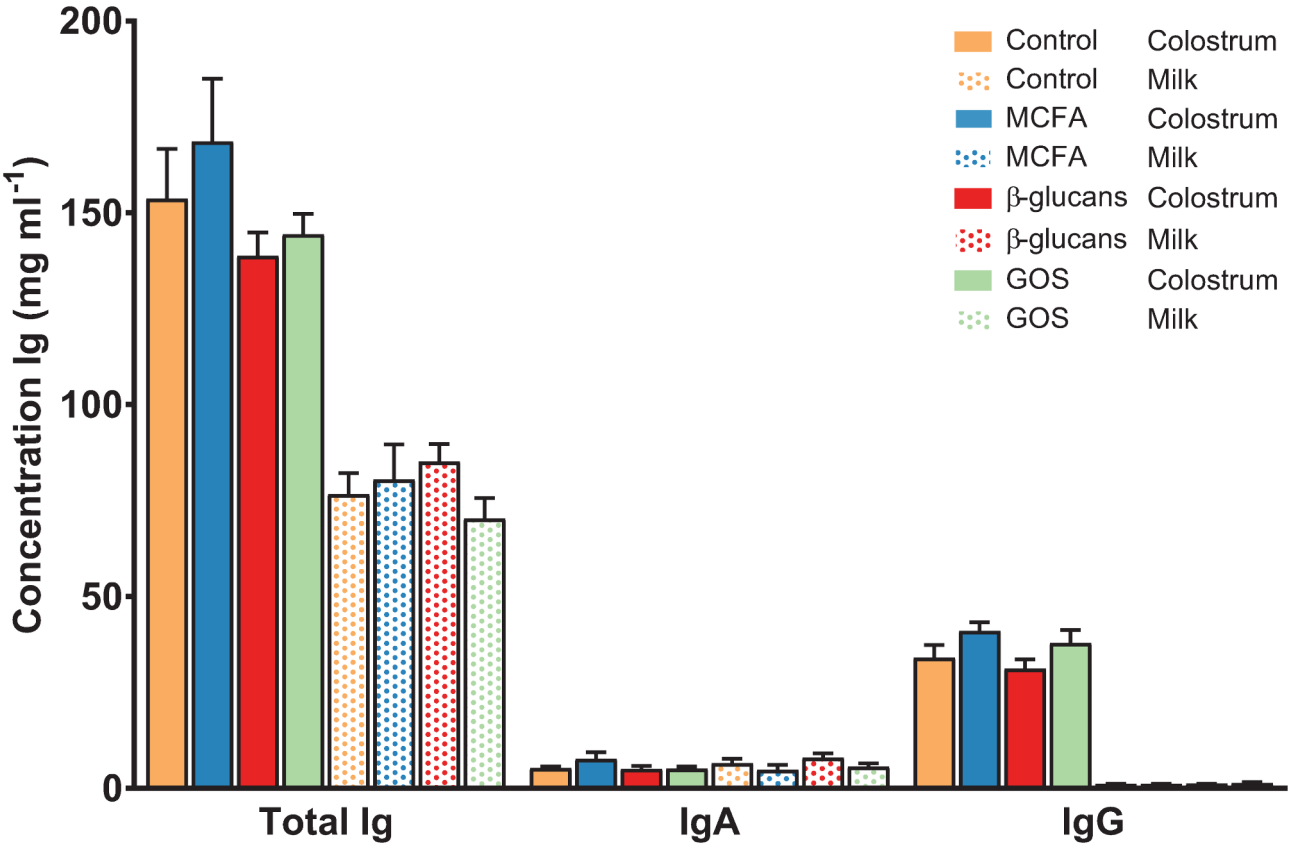

Fig. 1. Immunoglobulin concentration in colostrum and milk. Concentration of immunoglobulins in colostrum and milk (dotted bars) of sows that received a control feed (green), or a diet with either medium chain fatty acids (MCFA, blue), β-glucans (BG, magenta) or galacto-oligosaccharides (GOS, light green) in lactation feed ($P > 0.10$). Error bars depict the Standard Error of the Mean.

The effect of maternal dietary interventions on piglet gut microbiota and gene expression (treatment versus control groups)

When comparing the treatment groups with the control, both alpha and beta diversity revealed no significant differences, except for the offspring of maternal administration of MCFA (Table 1). On day 1, a significant increase (P value < 0.01) in observed species was found in the jejunum of the MCFA group compared to the respective control group in the maternal administration route (Table 1). Also, a trend (P value > 0.05 and < 0.1) was observed, i.e., a decrease in observed species at day 31 for MCFA treatment compared to the control group. For the beta diversity measures, no treatment effects were observed other than a trend at day 1 in colon (GOS vs. control) in, indicating a non-homogenously dispersion of the groups when comparing GOS to the control (Table 1). To get a general overview of important genera between the two time-points, i.e., d 1 and 31, as well as for each treatment, we have plotted the top 10 genus, and grouped all other genera in 'Other' (Fig. 2). To make it possible to compare the maternal and the neonatal route (result follow later), we have generated a list of the 10 most abundant genera when considering all these different time-points and dietary interventions. This resulted in the following top 10 across both routes and intervention (core microbiota); *Escherichia-Shigella*, *Clostridium* sensu stricto 1, *Actinobacillus*, *Streptococcus*, Bacteria, Chloroplast, *Lactobacillus*, *Turicibacter*, HT002, and Pasteurellaceae.

Next to the microbiota changes, the effect on host gene expression was examined. At day 1, this resulted in one differentially expressed gene (DEG) for MCFA treatment versus the control in the jejunum, and 816 colonic genes when comparing GOS treatment with the control group (Table 1). At day 31, all three treatments led to significant changes in host gene expression compared to the control sow diet group: 99 for MCFA, 21 for BG, and 77 for GOS (Table 1). Summarized, all three dietary interventions lead to differences in host gene expression, and it depended on the intervention diet what the exact effect was.

Pathway enrichment was performed for the different treatments on the day sufficient DEG were detected to serve as input for pathway analysis (Table 1). Analysis showed 64 significantly altered colonic epithelial pathways (corrected P value < 0.0001) for day 1 when comparing GOS treatment versus the control group. For clarity, Table 2 shows only the top 10 significant enriched pathways, where many pathways affected by GOS participate in immune related processes. Moreover, other significant pathways are linked to metabolic processes, i.e., MIF Mediated Glucocorticoid Regulation and Transport of Inorganic Cations/anions and Amino Acids/oligopeptides. For day 31, we observed ten significantly enriched pathways when comparing the MCFA treated

Day	Tissue	Treatment	Alpha diversity[1]			Beta diversity	
			Observed	Shannon	Pielou's evenness	adonis	Betadisper
1	Jejunum	**MCFA**	<u>< 0.01</u>	0.19	0.86	0.64	0.42
1	Ileum	**BG**	0.77	0.26	0.37	0.65	0.35
1	Colon	**GOS**	0.60	0.79	0.54	0.52	*0.06*
31	Jejunum	**MCFA**	*0.07*	0.13	0.25	0.35	0.71
31	Ileum	**BG**	0.71	0.62	0.58	0.33	0.45
31	Colon	**GOS**	0.29	0.19	0.20	0.43	0.34
Day	**Tissue**	**Treatment**	**# Of differentially expressed genes[2]**			**Up regulated**	**Down regulated**
1	Jejunum	**MCFA**	1			1	0
1	Ileum	**BG**	0			0	0
1	Colon	**GOS**	816[3]			463	353
31	Jejunum	**MCFA**	99[3]			62	37
31	Ileum	**BG**	21[3]			0	21
31	Colon	**GOS**	77[3]			36	41

Table 1. Results of alpha and beta diversity (top) and differential gene expression analysis (bottom) of offspring, with a diet intervention of the Sow, when comparing the treatment control combinations for the maternal administration route at day 1 and 31. Significant values are underlined, trends are italic. [1]Student's *t*-test—2-tailed, homoscedastic (assuming homogeneity of variance). [2]Adjusted P-value (False Discovery Rate) < 0.05 and logFC>| 2.0, [3]used as input for pathway enrichment analysis.

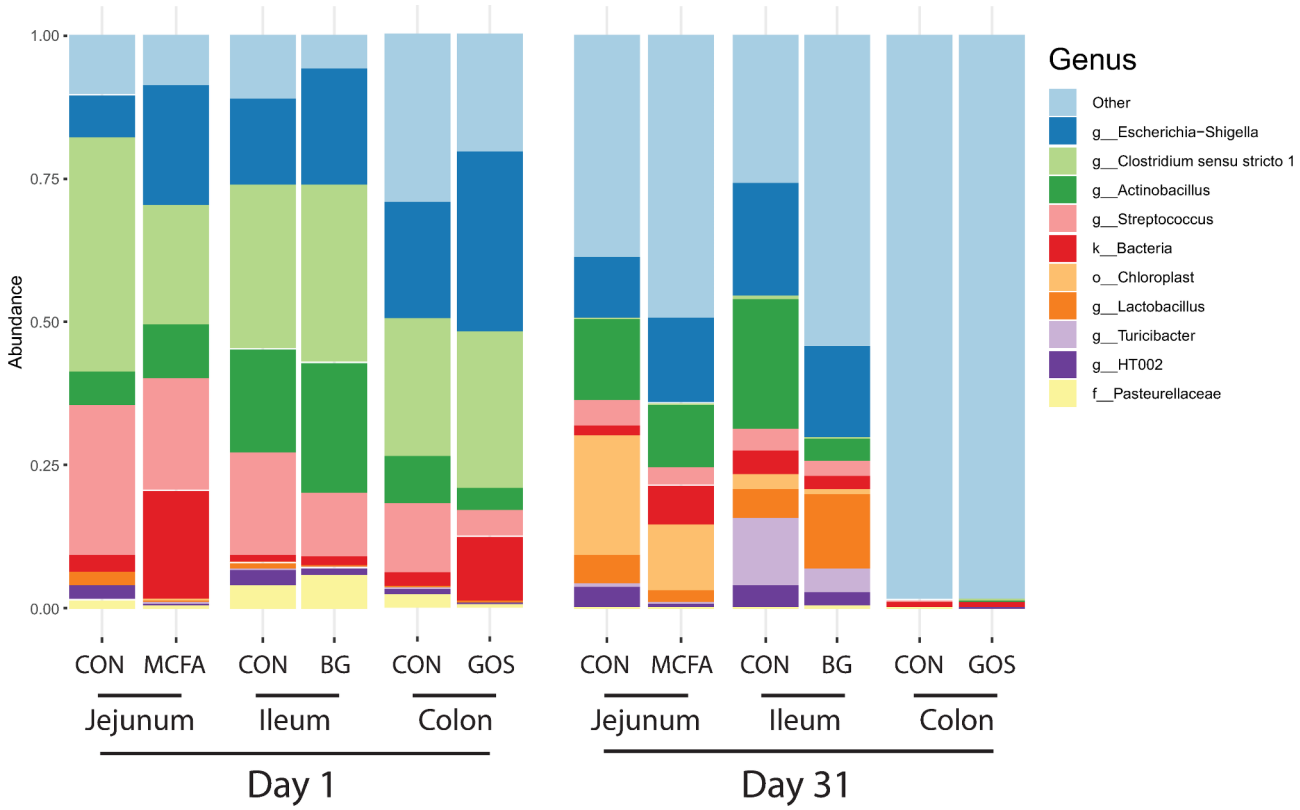

Fig. 2. The effect of maternal dietary interventions on the relative abundance of 10 most abundant genera in gut microbiota of piglets at d1 and 31. Average values per dietary interventions and time-point are displayed.

Day	Tissue | Treatment	Score[1,2]	'SuperPath' name
1	Colon | GOS	101.20	Innate Immune System
		51.42	Cytokine Signaling in Immune System
		50.19	NF-kappaB Signaling
		46.43	MIF Mediated Glucocorticoid Regulation
		43.89	Overview of Interferons-mediated Signaling Pathway
		42.29	Transport of Inorganic Cations/anions and Amino Acids/oligopeptides
		41.55	Chemokine Superfamily: Human/Mouse Ligand-Receptor Interactions
		39.45	Interferon Gamma Signaling
		39.17	Immunoregulatory Interactions Between a Lymphoid and A Non-Lymphoid Cell
		39.09	PAK Pathway
31	Jejunum | MCFA	27.71	Transport of Inorganic Cations/anions and Amino Acids/oligopeptides
		23.96	Proximal Tubule Transport
		22.36	PPAR Signaling Pathway
		20.57	Metabolism
		17.64	Statin Inhibition of Cholesterol Production
		16.35	Cell Adhesion_Cell-matrix Glycoconjugates
		15.88	Innate Immune System
		15.59	NF-kappa B Signaling
		14.68	Disorders of Transmembrane Transporters
		14.42	Visual Phototransduction
	Ileum | BG	15.58	Signal Transduction
		13.66	Sympathetic Nerve Pathway (Neuroeffector Junction)

Table 2. Significantly enriched pathways of the intestine of the offspring when diet interventions were applied in the Sow, when comparing the treatment control combinations for the maternal administration route at day 1 and 31. [1]The binomial distribution is used to test the null hypothesis that the user's input genes are not over-represented within any pathway. The presented score for each match is a transformation (−log2) of the resulting p-value, where higher scores indicate better matches. Results with p-values lower than 10–50 are assigned the maximum score. Corrected p-value smaller or equal to 0.0001. [2]For clarity, where applicable only the top 10 of significantly enriched pathways are shown.

group versus the control (jejunum), where three pathways were immune related, i.e., PPAR Signaling Pathway, Innate Immune System, and NF-kappa B Signaling. When comparing the BG group versus the control, (ileum) two significantly enriched pathways were observed of which one participated in signal transduction.

The effect of neonatal dietary interventions on gut microbiota and host gene expression (treatment versus control groups)

For the neonatal administration group of piglets, only day 31 could be analyzed since the dietary interventions start at day 1, after the intake of colostrum. No significant differences were observed when comparing the alpha and beta diversity of the microbiota of the neonatal administration groups versus the control group at day 31. Nevertheless, to get a general overview of important genera for each treatment on d 31, we have plotted the top 10 genus, and grouped all other genera in 'Other' (Fig. 3). As mentioned before, to make it possible to compare the maternal and the neonatal (result follow later) route, we have generated a list of the 10 most abundant genera when taking into account all these different time-points and dietary interventions. This resulted in the following top 10 across both routes and interventions (core microbiota); *Escherichia-Shigella*, *Clostridium* sensu stricto 1, *Actinobacillus*, *Streptococcus*, Bacteria, Chloroplast, *Lactobacillus*, *Turicibacter*, HT002, and Pasteurellaceae. However, gene expression of the intestine was altered as a result of neonatal administration in two groups, 94 genes were differentially expressed after MCFA supplementation, and 6 genes were altered when GOS was administered to the piglets, whereas BG did not alter gene expression compared to the control (Table 3). The pathway enrichment analysis resulted in four significantly enriched pathway (corrected P value < 0.0001) for MCFA and three significantly enriched pathway for GOS (Table 4). Pathways affected by neonatal intervention, GOS, participated in several pathways including signaling and cholesterol metabolism. For β-glucan administration no significantly enrichment of pathways was observed. The affected pathways by neonatal administration, i.e., MCFA and GOS, did not show overlap with pathways changed by maternal administration of feed additives, suggesting that the administration route plays a key role in the effect of a feed additive on gut functionality. This will be evaluated in more detail in the next paragraph.

Effect of administration route on microbiota and host gene expression at day 31, direct comparison of neonatal versus maternal route groups

To gain more insight into the different administration routes on microbiota and host gene expression, we directly compared both intervention routes. The experiment was performed at the same farm, using the same feeds,

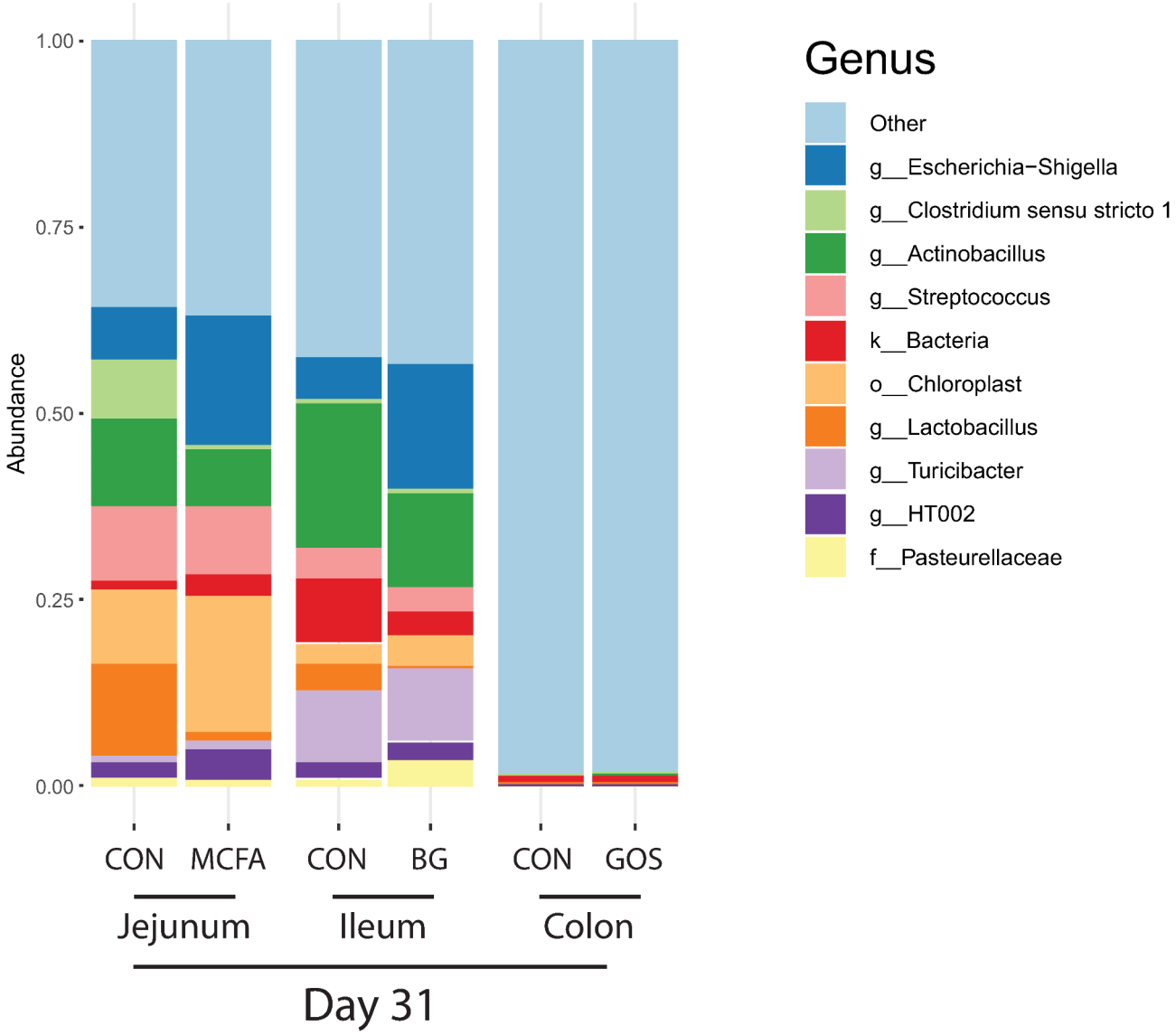

Fig. 3. The effect of neonatal dietary interventions on the relative abundance of 10 most abundant genera in gut microbiota of piglets at 31. Average values per dietary interventions are displayed.

Tissue	Treatment	Alpha diversity[1]			Beta diversity	
		Observed	Shannon	Pielou's evenness	adonis	betadisper
Jejunum	**MCFA**	0.19	0.77	0.30	0.52	0.68
Ileum	**BG**	0.11	0.27	0.41	0.29	0.84
Colon	**GOS**	0.16	0.30	0.60	0.61	0.10

Tissue	Treatment	# Of differentially expressed genes[2]	Up regulated	Down regulated
Jejunum	**MCFA**	94[3]	49	45
Ileum	**BG**	0	0	0
Colon	**GOS**	6[3]	2	4

Table 3. Results of alpha and beta diversity (top) and differential gene expression analysis (bottom) in piglets after diet intervention from day 1 after birth onwards, when comparing the treatment control combinations for the neonatal administration route at day 31. [1]Student's *t*-test—2-tailed, homoscedastic (assuming homogeneity of variance). [2]padj<0.05 and logFC>|2.0|, [3]used as input for pathway enrichment analysis.

Day	Tissue | Treatment	Score[1]	'SuperPath' Name
31	Jejunum | MCFA	18.81	Chemokine Superfamily: Human/Mouse Ligand-Receptor Interactions
		17.38	Cell Adhesion_Cell-matrix Glycoconjugates
		17.31	Integrin Pathway
		15.11	MIF Mediated Glucocorticoid Regulation
	Colon | GOS	16.01	Familial Hyperlipidemia Type 1
		15.92	Statin Inhibition of Cholesterol Production
		13.41	Plasma Lipoprotein Assembly, Remodeling, and Clearance

Table 4. Significantly enriched pathways of piglets after diet intervention from day 1 onwards when comparing the treatment control combinations for the neonatal administration route at day 31. [1]The binomial distribution is used to test the null hypothesis that the user's input genes are not over-represented within any pathway. The presented score for each match is a transformation (−log2) of the resulting p-value, where higher scores indicate better matches. Results with p-values lower than 10–50 are assigned the maximum score. Corrected p-value smaller or equal to 0.0001.

Day	Tissue	Treatment	Route	Alpha diversity[1]			Beta diversity	
				Observed	Shannon	Pielou's evenness	adonis	betadisper
D31	Jejunum	MCFA	Mat vs. Neo	0.03	0.12	0.21	0.25	0.59
D31	Ileum	BG	Mat vs. Neo	0.38	0.22	0.21	*0.07*	0.19
D31	Colon	GOS	Mat vs. Neo	*0.06*	0.04	*0.08*	0.02	0.92

Day	Tissue	Treatment	Route	# Of differentially expressed genes[2]	Up regulated	Down regulated
31	Jejunum	MCFA	Mat vs. Neo	515	251	264
31	Ileum	BG	Mat vs. Neo	503	221	282
31	Colon	GOS	Mat vs. Neo	996	628	368

Table 5. Results of alpha and beta diversity and differential gene expression analysis in intestine of piglets when comparing the administration routes (maternal and neonatal) within the dietary intervention. Significant values are underlined, trends are italic. [1]Student's *t*-test—2-tailed, homoscedastic (assuming homogeneity of variance). [2]padj<0.05 and logFC>|2.0|.

and time frame thus, excluding batch effects. Therefore, this allowed for a direct comparison of maternal versus neonatal administration for post-natal day 31. The distribution of gender in these two intervention groups was slightly biased (for details, see Table S3 in the material and methods section). To examine the effect of this bias, we first compared the respective control pigs between gender. No significant differences in observed species/richness or beta diversity were found between gender. We did observe significant changes in the Shannon index and Pielou's evenness (the count of individuals of each microbial species in an intestinal area, where 0 means no evenness and 1 means complete evenness) for the ileum at day 31 (Table S5), but not for jejunum and colon. Compared to the maternal control group, the neonatal control group showed a higher Shannon index (P value = 0.04), respectively 3.63 ± 0.87 versus 4.60 ± 0.51, where the Pielou's evenness (P value = 0.03) was 0.56 ± 0.07 versus 0.65 ± 0.05, respectively. Differential gene expression analysis showed that there were almost no differences between the control groups of the administration routes. The only DEG observed were at day 31, where one DEG (OSM) was found for ileum and six DEG (NEK11, SLITRK2, SMC6, KDM6A, LOC100157115, and LOC102168197) for colon (Table S5). Thus, gender and handling of piglets (neonatal route) only had a minor influence on the variables that we measured.

Subsequently, the maternal and neonatal administration routes of each dietary intervention were directly compared. Obviously, only for post-natal day 31 as the neonatal group intervention commenced at day 1. Here, we observed significant differences in alpha and beta diversity. At day 31, the observed species in jejunum (MCFA) was significantly higher (P value = 0.03) in the maternal administration route compared to the neonatal group, whereas for colon (GOS) a trend was observed and the effect in number of species was vice versa (P value = 0.06, Table 5 and Table S5). Furthermore, the Shannon index was also significantly (P value = 0.04) lower in the maternal administration route compared to the neonatal group for GOS treated animals. For the beta diversity, we observed a significant difference (P value = 0.02) for colon (GOS), and a trend (P value = 0.07) for ileum (BG) (Table 5 and Figs. S1, S2, S3). At day 31, DEG were observed for all three treatments, i.e., for jejunum (MCFA) 515, for ileum (BG) 503, and colon (GOS) 996 DEG (Table 5).

These identified DEG were used as input for pathway enrichment analysis to identify the biological activity. For jejunum (MCFA) 68 enriched pathways (corrected P value < 0.0001) were observed. In the top ten, seven pathways were linked to immune status and/or response (see Table 6). For ileum (BG), 8 enriched pathways were observed, which were mainly associated to metabolism. For colon (GOS) 41 enriched pathways were observed. In the top ten, two enriched pathways were associated to immune related processes, i.e., Innate Immune System, Statin Pathway—Generalized, Pharmacokinetics pathway (Table 6).

Tissue | Treatment	Score[1,2]	SuperPath Name
Jejunum | MCFA	133.46	Innate Immune System
	58.49	Overview of Interferons-mediated Signaling Pathway
	57.76	Cytokine Signaling in Immune System
	47.00	Allograft Rejection
	46.23	Chemokine Superfamily: Human/Mouse Ligand-Receptor Interactions
	45.87	MIF Mediated Glucocorticoid Regulation
	45.22	Interferon Gamma Signaling
	45.12	Metabolism
	39.90	NF-kappa B Signaling
	37.90	Immunoregulatory Interactions Between a Lymphoid and A Non-Lymphoid Cell
Ileum | BG	34.37	Metabolism
	18.33	One-carbon Metabolism and Related Pathways
	17.83	Transport to The Golgi and Subsequent Modification
	16.28	Metabolism of Proteins
	14.89	Synthesis of Substrates in N-glycan Biosynthesis
	14.89	Nuclear Receptors Meta-pathway
	14.73	Trans-sulfuration and One-carbon Metabolism
	13.68	Peptide Hormone Metabolism
Colon | GOS	81.52	Metabolism
	40.36	Innate Immune System
	31.38	Transport of Inorganic Cations/anions and Amino Acids/oligopeptides
	28.90	Nuclear Receptors Meta-pathway
	28.78	Statin Inhibition of Cholesterol Production
	23.92	Proximal Tubule Transport
	22.93	Statin Pathway - Generalized, Pharmacokinetics
	22.51	Ponatinib Pathway, Pharmacokinetics/Pharmacodynamics
	22.00	Metabolism of Water-soluble Vitamins and Cofactors
	21.75	Clomipramine Pathway, Pharmacokinetics

Table 6. Pathway enrichment results of intestinal segments for the comparison in administration route (maternal vs. neonatal) for the three dietary interventions at day 31. [1]The binomial distribution is used to test the null hypothesis that the user's input genes are not over-represented within any pathway. The presented score for each match is a transformation (−log2) of the resulting p-value, where higher scores indicate better matches. Results with p-values lower than 10–50 are assigned the maximum score. Corrected p-value smaller or equal to 0.0001. [2]For clarity, where applicable only the top 10 of significantly enriched pathways are shown.

Discussion

Based on previous published studies[15–18,22,23] and the presumed mode-of-action we examined for each feed additive a specific intestinal segment, where MCFA was studied in jejunum, BG in the ileum, and GOS in colon. Although these different segments exert their specific functions, generic biological processes will also occur, for example related to cell integrity, cell renewal, and to some extent immunological processes. Here, microbiota composition and host gene expression were used as proxies for possible effects on gut functionality and gut development. We compared the effect of route of administration of these additives via either sows' lactation feed or direct neonatal administration by oral gavage. Our results show that the type of intervention influences gut functionality, and in addition, that the administration route (maternal or neonatal) has a major effect on the outcome of these parameters. The chosen time-points reflect the direct effect of maternal interventions at d1, whereas d31 captures the accumulated effect over time, including the immediate post-weaning effect, both providing insights into the impact of dietary interventions during critical transition periods. A limitation of our study is the bias towards European weaning practices, as weaning age (here d28) was chosen to align with current practices in Europe. While this ensures relevance within the European context, it may not fully represent global variations in weaning practices. Additionally, our study focused on critical moments in the piglets' life, such as the immediate post-weaning period (d31). A longitudinal study would provide a comprehensive understanding of the long-term effects of dietary interventions. However, conducting such a study would require a significantly larger number of piglets, which poses challenges in terms of resources and adherence to the 3Rs (Replacement, Reduction, and Refinement) principles in animal research. Balancing scientific rigor with practical considerations is always a challenge, and we acknowledge that our study represents a snapshot rather than a complete longitudinal analysis.

Impact of feed additives when administered via Sow lactation feed

Sows were administered the feed additives, i.e., MCFA, BG, or GOS, in the last days of gestation. However, we did not observe any differences in the Ig levels in colostrum or milk when comparing intervention groups to the respective controls. Besides, the sow vaginal microbiota did not differ significantly either. This suggests that the offspring encountered similar maternal Ig factors and microbiota, independent of the dietary intervention of the sow. Hence, the differences in neonatal microbiota and host gene expression can be accounted to and altered milk composition other than immunoglobulins or, alternatively through a factor changing *in utero* environment. We observed limited significant differences for the microbiota effects due to the dietary intervention, where only in jejunum at day 1 a significant difference was observed in alpha diversity of the observed species after MCFA supplementation. Despite this significant shift in the observed species, only one DEG (INS gene) was observed in the jejunum at day 1 after intervention with MCFA. This phenomena of no to low correspondence between microbiota and host gene expression was observed throughout this study. This could be due the fact that we only examined the presence and abundance of the microbiota and did not look at their (putative) function. Because we investigated microbiome and gene expression at the same time point, we could not identify processes changing in time. To predict the microbial function, metagenomics can be used, whereas metatranscriptomics allows to measure the actual (collective) functionality of the microbiota[26]. This shows that follow-up studies are needed to better characterize and understand the dynamics between microbial colonization and host tissue development.

In colon, we observed a total of 816 DEG at day 1 for GOS. Enriched pathways were mainly involved in immune related processes, such as innate immunity and cytokine and chemokine signaling. This implies that GOS administration to sows might lead to altered colostrum/milk composition, affecting these pathways in the offspring. As our primary interest was in characterizing the response of the piglet regarding the intestinal functionality, we did not further analyze the colostrum and milk samples in detail. Nevertheless, other studies have shown that sow nutrition impacts the neonatal piglet survival[27,28]. In addition, long-term intake of resistant starch, being a relatively fast fermentable substrate, improves colonic mucosal integrity and reduces immune reactivity[29]. Since starches and GOS are both saccharides, we could expect similar functionality for GOS. Supplementation of pig diets with the polysaccharide laminarin showed an immunological effect in the colon as well, where several immune genes, including IL-6, IL-17 A, and IL-1β, were decreased in expression[30]. Our results of day 1 also underline the window-of-opportunity to modulate the piglet's intestinal functionality, where colostrum intake in the first 24 h is important for piglet survival[31], and simultaneously colonization of the intestinal microbiota occurs[32].

At day 31, alpha or beta diversity measures of the microbiota did not significantly change in all three feed additive groups. However, we did observe 99, 21, and 77 DEG, for MCFA, BG, GOS, respectively. Pathway analysis revealed significant pathways for MCFA in jejunum and, although to a lesser extent, for BG in ileum. For MCFA (jejunum), many of these enriched pathways were related to metabolism or immunological processes. The change in expression of genes related to metabolism was expected, as enterocytes can utilize MCFA directly for energy support and contribute to the barrier function of the intestine[33]. Another study showed increased villi length and decreased crypt depth after MCFA supplementation[34]. In addition to the direct impact on metabolism, MCFA also have antimicrobial and antiviral activity in gastric lining and the small intestine[35,36]. Inhibition of *Salmonella typhimurium* by MCFA has also been shown in an in vitro system of pig cecum[37]. Changes in biological processes have been observed already, for example increased intra-epithelial lymphocytes in jejunum after supplementing piglets with a mixture of MCFAs[38]. The link to immunological processes is more ambiguously in our study; only three pathways could be linked to immunity, i.e., PPAR signaling, innate immune system, and NF-kappa B signaling. For example, PPARγ is known to play a key role in the immune response by on one hand restricting inflammatory cytokines expression and on the other hand directing immune cell differentiation towards more anti-inflammatory phenotypes[39]. These results show that immune modulation is occurring due to the supplementation of MCFAs. For BG (ileum) significantly enriched pathways were related to signal transduction and nervous system. It has been reported that the gut microbiota can metabolize dietary fibers, i.e., BGs, to produce a range of neurotransmitters. These neurotransmitters stimulate the vagus nervus and consequently may modulate brain activity[40]. The signal transduction pathway is very generic and part of many biological processes, examples of such cellular responses to extracellular stimuli are gene activations and metabolism alterations. The observed DEG for BG suggest that BG are processed by the microbiome, generating neurotransmitters, which in turn alter gene expression of the host intestinal tissue, here ileum. Thus, maternal supplementation of feed additives mainly alters gene expression of neonates, and the effect depends on the additive and timepoint of sampling.

Impact of feed additives when administered directly to piglets in the neonatal stage

Oral dosing of feed additives did not result in statistically significant differences in alpha or beta diversity of the resident microbiota. Nevertheless, we did observe 94 DEG for MCFA in jejunum and six DEG (FABP2, MTTP, APOA4, RBP2, SMC6, and LOC100626041) for GOS in colon. Subsequent pathway analysis showed four significantly enriched pathways for MCFA: Chemokine superfamily, cell adhesion, the integrin pathway, and MIF mediated glucocorticoid regulation. These enriched pathways are all involved in the physical and immunological barrier function. Chemokines are a large family of small, secreted proteins that participate in movement of (immune) cells and the activation of an immune response. Moreover, cell adhesion is also an intricate aspect of these moving (immune) cells in the gut, whereas the integrin pathway could also be linked to this cell-cell and cell-extracellular matrix adhesion that is needed for the barrier function. The macrophage migration inhibitory factor (MIF) mediated glucocorticoids are known to be potent agents that regulate anti-inflammatory and immunosuppressive processes[41,42]. As mentioned in the maternal administration route paragraph, MCFAs exert antibacterial activity and can be taken up directly by enterocytes, as well as increase body weight gain in post-

weaned pigs and increased villus length and crypt depth in the small intestine[43]. Taken together, our results are in line with published studies that show an effect of MCFA on the physical and immunological barrier function of the small intestine in pigs.

For the GOS treatment we observed three significantly enriched pathways: Familial hyperlipidemia type 1, statin inhibition of cholesterol production, and plasma lipoprotein assembly, remodeling, and clearance. All three pathways participate in lipid metabolism. Therefore, our results suggest that the GOS treatment alters lipid metabolism of the colonic epithelium. Similar observations have been made in mice where GOS may improve lipid metabolism[44] as well as in pigs where pre-weaning supplementation of inulin decreased expression of genes in ileum involved in lipid metabolism[45]. These observations and our results support the idea that GOS influences lipid metabolism. However, additional research is needed to investigate to which extent GOS has this effect, since only six DEG were identified in our study. Summarizing, we did not measure an effect of BG after neonatal administration in the ileum at day 31, while GOS and MCFA altered local gene expression of host cells. An effect of BG would have been expected based on broad use in animal nutrition, however scientific proof in pigs may be limited[17]. Thus, the effect of direct diet intervention in piglets altered gene expression of the intestine at day 31 but did not significantly affect microbiome composition.

The administration route determines the effect of feed additives on piglet gut functionality

Through the comparisons described above, we have shown that the route of administration plays a key role in the effect a feed additive has. We therefore also directly compared the microbiota and gene expression profiles of the offspring between the neonatal and maternal administration routes for day 31. To assess the influence of stress by oral gavage and gender bias in the groups, we compared gene expression and microbiota in the different control groups. On microbiota parameters there was no effect, while one DEG was observed for BG (ileum) and six DEG for GOS (colon). Since these effects were minor, we continued and directly compared neonatal and maternal administration routes and identified a high number of DEG, ranging from 500 to 1,000 in each intervention group. When zooming in on the top ten of significantly enriched pathways for each feed additive, for MCFA immunological pathways were highly represented, for BG metabolism pathways were altered, and for GOS primarily metabolism processes and a few immunological pathways were changed. This implies that the combination of a feed additive and administration route has a substantial impact on the host response in the intestine. An additional observation is that the significance of the MCFA pathways is generally higher, with scores ranging from 38 to 134, whereas for GOS scores ranged from 22 to 82, and in BG scores range from 14 to 34. Hence, MCFA administration may lead to a more differentially orchestrated response when comparing the neonatal and maternal administration route then the other additives. Again, keeping in mind the difference in dosage applied. Such a well-orchestrated response could potentially be linked to the fact that all significantly enriched pathways are related to immunity. The effect of MCFAs is potentially greater when administered directly in the neonatal phase compared to maternal administration, this was strengthened by the fact that in our study the MCFA treatment in the neonatal administration the majority of DEG have higher expression compared to the maternal administration. For example, three DEG (i.e., S100A12, HMOX1, and EDARADD) were upregulated in the 'cytokine signaling in immune system' pathway after maternal administration, whereas 49 DEG were downregulated in maternal administration (Table 7). This underlines that the administration route, i.e., maternal or neonatal, matters for feed additives in pigs and their effect on immunological processes.

When interpreting these data, we must regard that for the two administration routes different dosages were used. In addition, maternal supplementation of feed additives is practically easier to implement compared to neonatal supplementation via oral gavage. We used oral gavage to make sure each animal was supplied similar amounts of the feed additive according to protocol, since it is not possible to obtain the correct dose per animal when supplying ad libitum creep feed to the piglets. Nevertheless, direct administration of these feed interventions in the neonatal period led to different gene expression responses in the offspring as compared to the (indirect) maternal administration route compared to the control group.

Conclusions and recommendations

Our study shows a distinct response is generated in terms of the intestinal functionality for each feed additive, i.e., medium chain fatty acids, β-glucans, and galacto-oligosaccharides, in combination with their specific matched intestinal segment. We observed differences in gene expression of the host tissues and to a lesser extent in microbiota diversity and composition. In addition, when comparing the maternal and neonatal administration route, a high number of differentially expressed genes and enriched pathways were observed that participated in processes that could be linked to intestinal barrier function, both physically and immunologically. Taken together, these results show that the combination of a feed additive and the route of administration affects the outcome regarding gut health of piglets and should be considered when designing new feed regimes. Furthermore, we argue that these insights are also directly relevant and useful to nutrition of human babies and infants, in view of the many similarities in physiology, immunity and gut function[46,47]. These pig models add to important and popular inbred rodent species living under specific pathogen free conditions (often 8–12 week old), since pig strains are outbred, and are raised under conventional conditions, providing an antigen-experienced immune repertoire including innate immune training[48] and broad T- and B-cell memory.

Materials and methods

Statement of ethical approval

The animal experiments described in this study were performed in strict accordance with the provisions of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (86/609 EG). The animal experiments were approved by the ethical committee Utrecht University

ID	Alias	Description	Function	logFC[1]
S100A12	Calgranulin-C	Protein S100-A12	Antimicrobial action	2.84
HMOX1	HSP32	Heme oxygenase 1	Cytokine signaling	2.56
EDARADD		EDAR associated death domain	Epithelial proliferation	2.09
IL1B		Interleukin 1 Beta	Important mediator of the inflammatory response, and participates in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.	− 2.01
CCL2		C-C motif chemokine 2	Chemotactic activity for monocytes and basophils	− 2.02
IL10		Interleukin 10	Has pleiotropic effects in immunoregulation and inflammation	− 2.03
MAOA		Amine oxidase [flavin-containing] A	Alternatively activated monocytes/macrophages	− 2.03
CD40		Tumor necrosis factor receptor superfamily member 5	Co-stimulation T and B cells	− 2.08
IL2RG	CD132	Interleukin 2 Receptor Subunit Gamma	Involved in the stimulation of neutrophil phagocytosis by IL15	− 2.08
OAS2		2'-5'-Oligoadenylate Synthetase 2	Pathogen recognition	− 2.09
HLA-B		Major Histocompatibility Complex, Class I, B	Antigen presentation	− 2.1
HLA-A		Major Histocompatibility Complex, Class I, A	Antigen presentation	− 2.16
FCGR1A		Fc Gamma Receptor Ia	Antigen signaling	− 2.17
ITGB2	CD18	Integrin beta	Key role in immune response and defects in this gene cause leukocyte adhesion deficiency	− 2.24
TNFSF13	CD256	Tumor necrosis factor ligand superfamily member 13	Important for B cell development	− 2.24
CD4		CD4 Molecule	T helper cells	− 2.25
IL10RA	CD210	Interleukin-10 receptor subunit alpha	Inhibits the synthesis of proinflammatory cytokines	− 2.26
GSTO1		Glutathione S-transferase omega-1	Involved in the metabolism of xenobiotics and carcinogens	− 2.27
ISG20	CD25	Interferon stimulated exonuclease gene 20	Involved in defense response to virus; negative regulation of viral genome replication; and nucleobase-containing compound catabolic process	− 2.3
IRF7		Interferon Regulatory Factor 7	Plays a critical role in the innate immune response against DNA and RNA viruses	− 2.42
HLA-DQA1		HLA class II histocompatibility antigen, DQ alpha 1 chain	Antigen presentation	− 2.48
USP18		Ubl carboxyl-terminal hydrolase 18	Involved in the negative regulation of the inflammatory response	− 2.5
STAT1		Signal transducer and activator of transcription 1	Intracellular signaling	− 2.58
IL18		Interleukin-18	Pro-inflammatory cytokine primarily involved in epithelial barrier repair, polarized T-helper 1 (Th1) cell and natural killer (NK) cell immune responses	− 2.59
BST2	CD317	Bone Marrow Stromal Cell Antigen 2	May play a role in pre-B-cell growth	− 2.69
HLA-DRB3		Major Histocompatibility Complex, Class II, DR Beta 3	Antigen presentation	− 2.74
GBP2		Guanylate Binding Protein 2	Providing broad host protection against different pathogen classes	− 2.78
IFNB1		Interferon beta	Defense against viral infections	− 2.78
HLA-DRA		Major Histocompatibility Complex, Class II, DR Alpha	Antigen presentation	− 2.86
IL27	IL30	Interleukin-27 subunit alpha	Regulate T-helper cell development, suppress T-cell proliferation, stimulate cytotoxic T-cell activity, induce isotype switching in B-cells	− 2.95
TRIM5	RNF88	Tripartite Motif Containing 5	Pathogen recognition	− 3.03
PSMB9		Proteasome 20 S Subunit Beta 9	An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides	− 3.06
TNFSF13B	CD257	TNF Superfamily Member 13b	Cytokine	− 3.11
CCR5		C-C chemokine receptor type 5	Co-receptor for macrophage-tropic virus	− 3.15
CCL4L1		C-C Motif Chemokine Ligand 4 Like 1	Chemokine that induces chemotaxis of cells expressing CCR5 or CCR1	− 3.2
IFIT3	P60	Interferon-stimulated protein 60	Positive regulation of apoptotic process; and response to virus.	− 3.22
TNFRSF17	CD269	TNF receptor superfamily member 17	Preferentially expressed in mature B lymphocytes, and may be important for B cell development and autoimmune response	− 3.23
IFITM1	CD225	Interferon-induced transmembrane protein 1	Restricts cellular entry by diverse viral pathogens	− 3.35
MX1		Interferon-induced GTP-binding protein Mx1	Antagonizes the replication process of several different RNA and DNA viruses	− 3.39
IFIT5	P58	Interferon Induced Protein with Tetratricopeptide Repeats 5	Positive regulation of apoptotic process; and response to virus.	− 3.4
PSMB8		Proteasome 20 S Subunit Beta 8	An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides	− 3.51
NOS2		Nitric oxide synthase, inducible (Fragment)	Enzymes generating reactive oxygen species	− 3.7
GSTA2		Glutathione S-transferase A2	Detoxification of electrophilic compounds,	− 3.82
CD86		CD86 molecule	Costimulatory signal for T cell proliferation and IL2 production	− 3.86
ISG15		Ubiquitin-like protein ISG15	Chemotactic activity towards neutrophils, direction of ligated target proteins to intermediate filaments, cell-to-cell signaling, and antiviral activity during viral infections	− 3.89
Continued				

ID	Alias	Description	Function	logFC[1]
MX2		Interferon-induced GTP-binding protein Mx2	Antimicrobial action	−3.9
HERC5		HECT and RLD domain containing E3 ubiquitin protein ligase 5	Acts as a modulator of the antiviral immune response	−4.08
IFIT2	P54	Interferon-induced protein with tetratricopeptide repeats 2	Positive regulation of apoptotic process; and response to virus.	−4.36
CCL5		C-C motif chemokine	Chemoattractant for blood monocytes, memory T-helper cells and eosinophils	−4.69
CXCL10		C-X-C motif chemokine 10	Pro-inflammatory cytokine	−4.78
CCL19		C-C motif chemokine	Normal lymphocyte recirculation and homing	−5.2
OASL		2′-5′-oligoadenylate synthase-like protein isoform a	Enables DNA binding activity and double-stranded RNA binding activity	−7.41

Table 7. Differentially expressed genes of maternal versus neonatal administration route of MCFA treatment that were observed in the cytokine signaling in immune system pathway. [1]log Fold Change.

according to the Dutch law on animal experimentation under registration number 2012.III.05.041 and in accordance with the ARRIVE guidelines (https://arriveguidelines.org).

Animal experiment

A schematic overview of the entire animal experiment is given in Fig. 4. This design was primarily designed to investigate the interventions within an administration route. However, the number of sows and piglets enrolled in this study was reduced by sharing the control group of sows and their offspring between the administration routes in order to adhere to the 3R principles (https://nc3rs.org.uk/who-we-are/3rs).

Thirty-nine Hypor sows (Hendrix Genetics, Boxmeer, The Netherlands) were housed at Trouw Nutrition's Swine Research Centre (Sint Anthonis, The Netherlands) in gestation group housing. Approximately one week prior to expected farrowing, sows were moved to the farrowing rooms. Sows had free access to water and were fed basal lactation diet without feed additives (Table S1) according to a standard feeding scheme allowing for an intake close to *ad libitum*. Feed intake was recorded using automated feeders in one of the departments. In the other departments, sows were fed twice daily by hand. Sow body weight was determined before entering the farrowing room and at weaning (d28). As this study was conducted in Europe (the Netherlands), our study aligned with local practices, typically weaning the piglets at around 28 days. Do note, that the primary focus was on administration routes of dietary interventions, not optimal weaning age. Farrowing was only induced in sows that did not farrow on day 114 of gestation. Cross-fostering of piglets was minimized and only occurred within the first 24 h within study groups. Cross-fostered piglets were excluded for further analyses within the experiment. When sows or piglets have received any medication, we have removed them form downstream analyses.

The impact of three feed additives on gut functionality of piglets was determined. Feed additives were either administered directly to neonatal piglets, or indirectly via maternal lactation feed. Vaginal swabs were collected from the sows 5 days before farrowing (before lactation diet was supplied), at farrowing and at weaning (d28). Colostrum (15 ml) was taken from sows immediately after the first piglet was born, or as soon as possible after the first piglet was born. Milk samples (15 ml) were taken after weaning (d28), day 31. Both samples were taken after cleaning the teat with alcohol to prevent microbiological skin contamination of the sample. The neonatal and the maternal administration route of feed additives were administered to one batch of sows and assessed in one experiment.

Feed additives

The effect of three feed additives on gut functionality was determined for medium-chain fatty acids (MCFA), β-glucans (BG), and galacto-oligosaccharides (GOS). MCFA consisted of a 1:1 ratio of oils containing 98% C10 (Capric acid) and 98% C12 (Lauric acid), both as monoglycerides and supplied by Greenvalley (Wageningen, The Netherlands). BG were provided in the form of Macrogard supplied by ORFFA (Breda, The Netherlands). This product contains at least 60% β-glucans in the form of cell walls from *Saccharomyces cerevisiae*. GOS was provided in the form of Vivinal GOS provided by Friesland Campina (Wageningen, The Netherlands). The product consists of a GOS rich whey product containing 68% galacto-oligosaccharides and 23% lactose.

Grouping and administration route of feed additives

Sows were allocated to one of three groups: maternal administration, neonatal administration, or the control group (Table S3). Litters were randomly allocated to the intervention groups and balanced for parity of the sow as well as for day of farrowing (neonatal administration route) or for body weight of the sow (maternal administration route). The sows in the control group were shared between the neonatal and maternal administration route.

In the neonatal group, feed additives were administered to the piglets using an oral drench of 2 ml, from day of birth until weaning (d28). 0.12 g of MCFA mixture, 45 mg of β-glucans or 1.2 g of GOS was supplied to each piglet per day (birth to weaning (d28)). The female piglets of the control group received a 2 ml oral drench containing demi-water at the same time-points. Doses were divided into two oral drenches during weekdays and given in one oral drench during weekends. Piglets received only sow's milk until 3 days before weaning. In the period from 3 days before weaning until 9 days after weaning, piglets were fed a weaner diet (Table S4).Our study aimed to establish a basal understanding of dietary interventions where we did not adjust for the weight

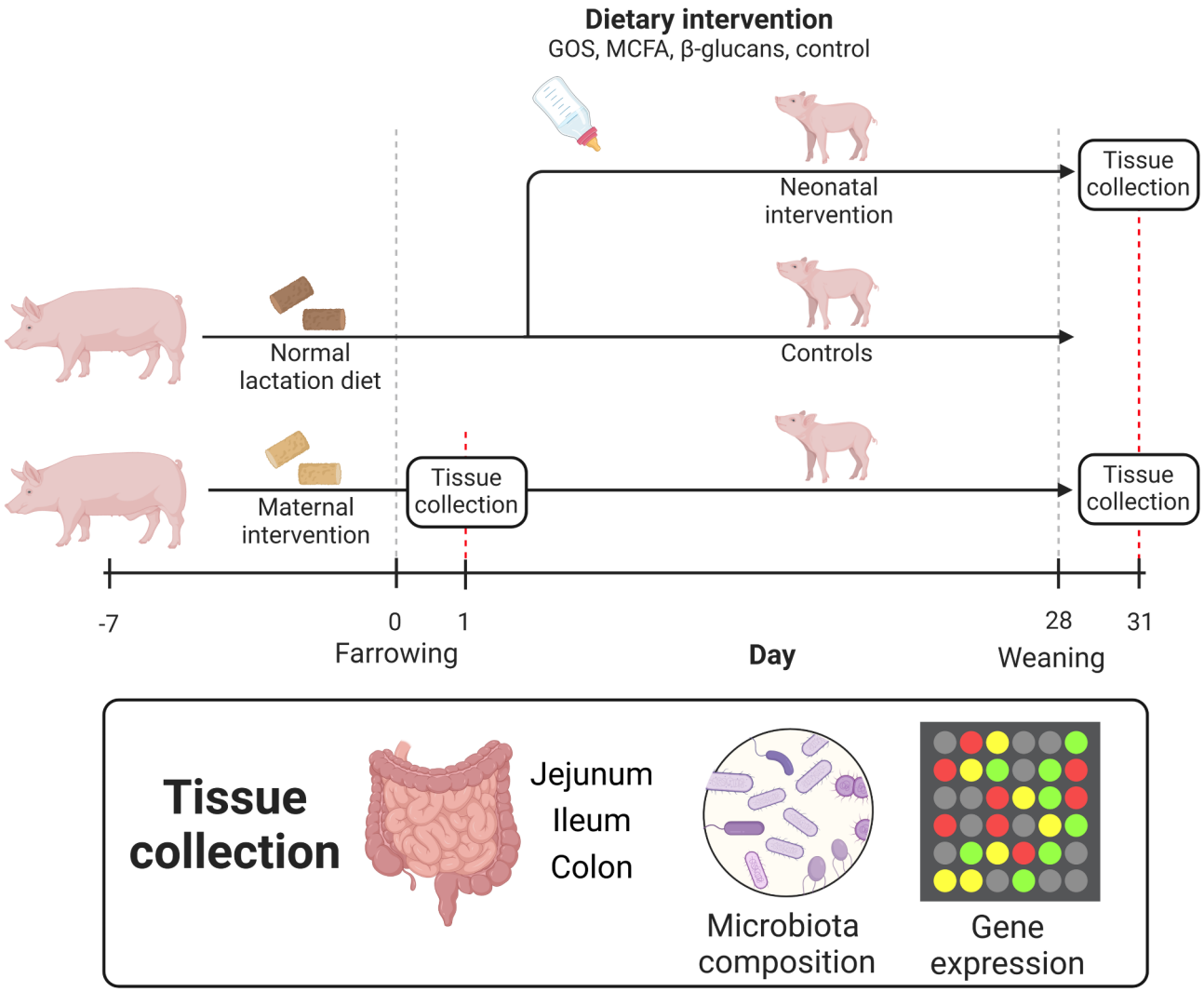

Fig. 4. Design of the animal experiment. Sows and piglets were divided into two groups, maternal and neonatal intervention. The maternal intervention group received a dietary intervention (MCFA, BG, GOS) via lactation feed starting 5 days prior to the expected date of farrowing. In the neonatal group, piglets received a single intervention via oral gavage per day after birth until weaning (d28). Piglets and sows in the control group received demi water. Piglets were sacrificed at post-natal day 1 and day 31 after birth, and gut tissue segments were collected to examine microbiota composition and host gene expression (visualized for day 31 only).

gain of the piglets. Nevertheless, this same rapid growth rate of suckling piglets makes daily weighing and dose adjustments impractical and stressful, so with future research needed to explore dynamic dosing strategies for more accurate application.

For maternal dietary intervention, feed additives were included in the lactation feed at the moment the sows entered the farrowing room until weaning (d28), in the following concentrations: 0.2% (2 g/kg feed) of MCFA mixture, 0.1% (1 g/kg feed) of β-glucans and 0.17% (1.7 g/kg feed) of GOS. The control group received the basal lactation diet without feed additives. The doses of different additives for piglets and sows were determined based on previous experiences, literature[49–54], and a pilot experiment (data not shown). These preliminary studies provided a foundation for selecting appropriate dosages that are both palatable and acceptable to the animals. We ensured that the chosen doses were within a range that would not adversely affect the health or growth of the piglets and sows.

Sampling and data collection of piglets

Piglets with visual signs of disease at the moment of allocation (sampling time) were excluded from sampling. Because we sampled from a larger cohort of animals at the farm, only clinically healthy piglets were considered for the study. General health of all piglets, as well as mortality and morbidity, was inspected and recorded daily. Piglet' body weight was determined at birth, and one day before weaning.

At day 1 after birth and 3 days post-weaning (day 31), piglets of the maternal administration route were sacrificed for necropsy and sample collection. For both days, seven piglets per treatment group and four control piglets were sacrificed. Whereas piglets from the neonatal group were sacrificed at day 31 for necropsy and sample collection, i.e., eight piglets per treatment group and twelve control piglets were sacrificed. Piglets were sacrificed by exsanguination after intravenous anesthesia with 24 mg kg^{-1} bodyweight of Euthasol (AST Farma, The Netherlands). During necropsy, samples from jejunum, ileum and mid-colon were collected for analysis of microbiota composition and gene expression of tissue. Digesta was collected and snap frozen on dry ice for microbiota analysis. Mucosal scrapings were collected and snap frozen for gene expression analysis.

Milk analysis

Immunoglobulins in colostrum and milk

The protein concentration of the in the samples was measured by the Bradford method (DC Protein assay KIT 2, Biorad) before and after precipitation of immunoglobulins (Ig) using polyethylene glycol. The Ig concentration was determined by subtracting the total protein concentration to the concentration of protein after Ig precipitation. The milk and colostrum samples were diluted to 1/100 for the total protein determination, and to 1/50 for the protein after precipitation determination.

IgA and IgG concentrations were determined with a double antibody sandwich commercial ELISA using HRP and TMB (Abnova KA2038 for IgA; Abnova KA2016 for IgG). Colostrum and milk samples were diluted to 1/50 000–1/100 000 and assayed in duplicate following the protocol supplied with the kit.

Analysis of microbiota composition

DNA isolation

Microbiota composition was determined of piglet' digesta from jejunum, ileum, and colon, and from vaginal swabs of sows. Samples were frozen on dry-ice after collection and stored at −80 °C. To isolate DNA, samples were mixed in a 1:1 ratio with phosphate buffered saline (PBS) and centrifuged for 5 min at 4 °C at 300×g. Supernatant was collected and centrifuged for 10 min at 4 °C at 9,000×g. DNA was extracted from the pellet using the "QIAamp FAST DNA stool minikit" according to manufacturers' instructions. Quality and quantity of DNA was checked using the NANOdrop (Agilent Technologies).

Sequence analysis of 16 S rDNA

PCR was used to amplify the 16 S rDNA V3 fragment using forward primer V3_F (CCTACGGGAGGCAGC AG) and reverse primer V3_R (ATTACCGCGGCTGCTGG). PCR conditions were as follows: 2 min at 98 °C, 15 × (10 s at 98 °C, 30 s at 55°C, 10 s at 72 °C), 7 min at 72 °C. PCR efficiency was checked on agarose gel by visual inspection. Sequence processing and statistical analyses were performed in R 3.6.1. (R Core Team, 2020). The amplicon sequences were demultiplexed per sample and subsequently filtered, trimmed, error-corrected, dereplicated, chimera-checked, and merged using the DaDa2 package (v.1.16.0[55]). By using the standard parameters except for *TruncLength*=(270,220), *trimLeft*=(25,33) and *minOverlap* = 10, and reads were classified against the SILVA v.138 database[56].

Statistical analysis of microbiota data

The phyloseq object creation and statistical analyses were performed in R 4.1.0, the associated rds object (MicrobiotaDataPiglets.rds) is online available doi: https://doi.org/10.5281/zenodo.7835159. The biodiversity of the microbiota was calculated by the vegan package http://cran.r-project.org/web/packages/vegan/ [57], by employing the Shannon diversity indices, as well as species and evenness. The Redundancy analysis (RDA) was also performed by using the vegan package. The following model was used on the family level microbiota data:

$$y = \text{Time (day)} + \text{Intervention} + \text{Time} * \text{Intervention} + \text{error} \quad (1)$$

Furthermore, statistical significance testing for over- and under-representation of the bacterial groups was made at the phylum / genus level by performing the Wilcoxon signed-rank test, and P-values were also converted to false discovery rate (FDR) values to correct for multiple testing.

Gene expression of intestinal tissue

RNA isolation

Total RNA was extracted from 50 to 100 mg of mucosal scraping of jejunum, ileum, or colon tissue. Samples were homogenized using the TissuePrep Homogenizer Omni TP TH220P in 5 ml TRIzol reagent (Life Technologies). The homogenate was centrifuged for 5 min at 21,000 × g. 350 µl of supernatant was used to isolate RNA using the Direct-zol kit (Zymo Research) according to instructions of the manufacturer. Quality control was performed on the BioAnalyzer (Agilent Technologies), quantity of RNA was determined using the TapeStation (Agilent 2200 TapeStation, Agilent technologies).

Microarrays, labelling and hybridization procedure

Labelling of RNA was done as recommended by Agilent Technologies using the One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labelling. 200 ng of total RNA was used as input, 600 ng of labelled cRNA was used to hybridize the porcine microarray (Agilent Technologies). Hybridization was performed at 65 °C for 17 h with head-over-head rotation. Microarrays were washed as recommended by the manufacturer. Microarrays were scanned using the Surescan high resolution scanner (Agilent Technologies) at a resolution of 3 µm, 20 bits and PMT of 100%. Feature extraction was performed using protocol 10.7.3.1 (v10.7) for 1 color gene expression.

Microarray data analysis

The data were analyzed using R (v3.0.2) by executing different packages, including LIMMA and arrayQualityMetrics[58]. The R package LIMMA from Bioconductor was used to correct for background (method="normexp" and offset = 1)[59]. Quantile normalization of the data was done between arrays. The duplicate probes mapping to the same gene were averaged ('avereps') and subsequently the lower percentile of probes were removed in a three-step procedure: (1) select the highest of the dark spots to acquire a base value, (2) multiply by 1.1, (3) the gene/probe had to be expressed in each of the samples in the experimental condition. To evaluate the differences between the experimental group (i.e., control versus intervention group), the contrasts between control and intervention group were studied for each time-point separately within the LIMMA package, including multiple testing correction. Microarray data were normalized and filtered. Subsequently, the cut-off for differential gene expression was set on a fold change of 2 with a probability of 95% (p value ≤ 0.05). Only annotated genes were included for further analysis.

Pathway enrichment analysis

The data were analyzed using GeneAnalytics[60] (© LifeMap Sciences 2022, v5.12 Build 767). Because human genes are better annotated and more information in different databases is available for humans than for pigs, a human background was used for the functional analyses[61]. From GeneAnalytics output files, pathways were retrieved with a high enrichment score (corrected p-value smaller or equal to 0.0001). To generate these scores, the binomial distribution was used to evaluate the null hypothesis that the user's input genes are not over-represented within any pathway. The presented score for each match is a transformation ($-\log_2$) of the resulting p-value, where higher scores indicate better matches. Results with p-values lower than 10^{-50} were assigned the maximum score.

Data availability

The phyloseq object creation and statistical analyses were performed in R 4.1.0, the associated rds object (MicrobiotaDataPiglets.rds) is online available doi: 10.5281/zenodo.7835159. The raw data is available under accession number PRJNA973638. Gene expression data was deposited in Gene Expression Omnibus under accession number GSE229947.

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Author contributions

Conceptualization, AdG, DS, and JMJR; methodology, AB, DS, AdG, and JMJR; software, AB and DS; formal analysis, AdG, AB, and DS; investigation, DS, MHvE, JGA, AdG, JMJR; writing—original draft preparation, AdG and DS; writing—review and editing, DS, MHvE, AdG, JMJR, HvH; visualization, AdG and DS; supervision, AdG and JMJR. All authors have read and agreed to the published version of the manuscript.

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Declarations

Ethics approval and consent to participate

The animal experiments described in this study were performed in strict accordance with the provisions of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (86/609 EG). The animal experiments were approved by the ethical committee Utrecht University according to the Dutch law on animal experimentation under registration number 2012.III.05.041 and in accordance with the ARRIVE guidelines (https://arriveguidelines.org).

Competing interests

The authors declare no competing interests.

Additional information

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