

RESEARCH ARTICLE

Prenatal caprine milk oligosaccharide consumption affects the development of mice offspring

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Scope: The composition of the gastrointestinal (GIT) microbiota, particularly in early life, influences the development of metabolic diseases later in life. The maternal microbiota is the main source of bacteria colonising the infant GIT and can be modified by dietary prebiotics. Our objective was to determine the effects of prenatal consumption of prebiotic caprine milk oligosaccharides (CMO) on the large intestine of female mice, milk composition, and offspring's development.

Methods and results: C57BL/6 mice were fed either a control diet, CMO diet, or galacto-oligosaccharide diet from mating to weaning. From weaning, some pups nursed by CMO, GOS, and control-dams were fed the control diet for 30 days. CMO or GOS-fed dams had increased colon length and milk protein concentration compared to control-fed dams. At weaning, pups from CMO-fed dams had increased body weight and colon length and increased proportions of colonic *Bifidobacterium* spp compared to the pups from control-fed dams. Thirty days after weaning, pups from CMO-fed dams had increased visceral fat weight compared to pups from control-fed dams.

Conclusion: Consumption of CMO by the dams during gestation and lactation improved the development of the pups, and the relative abundance of bifidobacteria and butyric acid in the colon, at weaning.

Keywords:

Caprine milk oligosaccharides / Development / Prebiotic / Pregnancy / Prenatal diet



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1 Introduction

The composition of the microbiota, particularly in early life, profoundly influences the development and maturation of the infant mucosal immune system [1] and metabolism [2]. A higher risk of metabolic diseases in adults, for example, has

been associated with changes in the GIT microbiota in early life [2]. The maternal microbiota is the main source of bacteria colonizing the infant GIT during labor [3] and breast feeding [4]. Emerging evidence suggests that these bacteria may also colonise the infant GIT before birth [5, 6]. The manipulation of the maternal microbiota (in humans and rodents) through antibiotics [7], diet [8], and prebiotic intake [9, 10] has been shown to affect the microbiota transmitted to the offspring both quantitatively and qualitatively and offspring development [11]. Therefore, the period from pregnancy to weaning is likely to be a critical window of opportunity for modifying the maternal GIT microbiota for improved infant development and long-term health benefits.

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Abbreviations: CMO, caprine milk oligosaccharides; CMOF, caprine milk oligosaccharide enriched fraction; GIT, gastrointestinal tract; GOS, galacto-oligosaccharide

Caprine milk has oligosaccharides structurally similar to human milk oligosaccharides [12], known to stimulate the development, maturation, and colonization of the neonate's GIT [13]. Important differences in the profile of goat and milk oligosaccharides were, however, also described [14, 15]. The ratio of fucosyl-oligosaccharides, for example, is high in human milk oligosaccharides, while this is extremely small in caprine milk. Human milk sialyl-oligosaccharides contain only the monosaccharide Neu5Ac, while the ratio of Neu5Ac/Neu5Gc in caprine milk is 36/64. In human milk the type 1 oligosaccharides, which contain Gal(β 1-3)GlcNAc, predominate over the type 2, which contain Gal(β 1-4)Glc, while caprine milk contains only type 2 oligosaccharides [14, 15]. The impact of these different milk oligosaccharides profiles on the GIT microbiota and host physiology have been poorly explored.

We have previously shown that a caprine oligosaccharide-enriched fraction (CMOF) supported the growth of selected bifidobacterial strains isolated from breast-fed infants, and stimulated *in vitro* production of acetate and lactate [16]. Oliveira et al. (2012) [17] has also demonstrated the prebiotic activity of CMOF in a batch culture system inoculated with adult human feces. The aim of this study was to determine the effects of prenatal consumption of CMOF on the colon microbiota and milk composition of the dams, and the offspring's development. In this study, we also examined whether the effects of maternal diet on pups' development persisted after the pups' diet was switched to the control diet for 30 days after weaning.

2 Materials and methods

2.1 Animals and study design

Animal experimentation was approved by AgResearch Grasslands Animal Ethics Committee (AE12579), in accordance with the New Zealand Animal Welfare Act 1999, New Zealand. Sixty three C57BL/6 mice (42 female and 21 male) were obtained from the AgResearch Ruakura Small Animal Facility and housed in a temperature and humidity controlled facility with a 12-h light/dark cycle. After acclimatization, 9-week-old mice were randomly assigned to groups of two females and one male and fed one of the following diets; AIN-76A (control diet), AIN-76A supplemented with 1% GOS (GOS diet), or AIN-76A supplemented with CMOF containing 1% caprine milk oligosaccharides (CMO diet). Diets were formulated by Research Diets, Inc. (NJ, USA). The CMO diet also contained 0.2% of GOS and other sugars as a result of the method used to obtain the CMOF (Table 1). CMOF was obtained by the method previous described in [18]. In short, caprine milk whey was processed by a combination of ultrafiltration, enzymatic hydrolysis of the lactose, solid-phase extraction, and rotary vacuum evaporation. Oligosaccharide characterization and quantification was performed on a Thermo Scientific LTQ XL-Linear Ion Trap Mass

Table 1. Composition of diets and caprine milk oligosaccharide enriched fraction

Ingredients ^{a)}	Control diet (AIN76A)	GOS diet	CMO diet
	Grams	Grams	Grams
Casein	200	200	200
DL-Methionine	3	3	3
Corn Starch	150	500	500
Maltodextrin	0	150	150
Sucrose ^{b)}	500	0	0
Cellulose, BW200	50	50	50
Corn oil	50	50	50
Mineral Mix S10001	35	35	35
Vitamin Mix V10001	10	10	10
Choline bitartrate	2	2	2
Composition of CMOF	Components	grams	grams
	Protein		0.014
	GOS	10	2
	Lactose	3.34	3.34
	Glucose	10	10
	Galactose	6.68	6.68
	Oligosaccharide ^{c)}		11
	Calcium		0.028
	Magnesium		0.014
	Potassium		0.377
	Sodium		0.21
	Iodine		0.0001
	Selenium		0.000001
	Total	1000.00	1022.02
			1033.6

a) All ingredients of the AIN-76A, GOS and CMO diet (except CMOF, sourced from New Zealand, and GOS, sourced from Yakult, Japan), were supplied by Research Diets.

b) The sucrose concentration was adjusted in the CMO and GOS diets to balance the energy and nutritional content of the AIN-76A diet.

c) Caprine milk oligosaccharides and their abundance: (13%) 3'- and/or 6'-galactosyl-lactose, (27%) 3'- and/or 6'-sialyl-lactose, (32%) 6'-glycolyl-neuraminyl-lactose, (9%) lacto-N-hexaose, (11%) disialyl-N-lactose, (8%) 6'-N-acetyl-L-glucosaminyl-lactose.

Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with electrospray ionization in negative mode using appropriated standards. Other sugars (lactose, galactose and glucose) were characterized and quantified using high performance liquid chromatography connected to a Thermo Hypercarb column. More information on diet composition and analysis is provided in Supporting Information Table 1.

Animals were mated for 11 days. After delivery, pups remained with their dams up to weaning (21 days) where dams and some of the pups were euthanized and sampled. To determine the long-term effects of maternal diet, the remaining pups were fed the control diet for 30 days, then euthanized and samples collected.

2.2 Sampling and analysis

2.2.1 Milk sample collection and nutrient composition

At days 5, 12, and 19 of lactation, mice were anesthetized with 0.2 mL of ketamine/xylazine/acepromazine mix (50 mg/mL ketamine, 5 mg/mL xylazine, and 0.5 mg/mL acepromazine), then injected with oxytocin (0.15 iu/mouse). Milking was done manually into a sterile tube and samples obtained for each mouse on each of the three sampling occasions were pooled prior to compositional analysis.

The dry matter and crude fat were determined using a method described by Gor et al. [19]. In short, dry matter was measured in triplicate on 10 μ L of milk in precombusted and preweighed tin capsules. After weighing, the samples were dried at 55°C for 5 h, cooled to room temperature, and reweighed, and the dry matter calculated. Total fat was measured using 100 μ L of milk which was transferred to a 20 mL glass tube and diluted ten times with Milli-Q water. Two hundred microliters of NH₃ solution (25%), 1 mL ethanol (100%), 3 mL diethyl ether, 3 mL petroleum ether (40–60°C), and 800 μ L Milli-Q water were added, with 30-s vortexing after each step. The sample was then centrifuged at 3000 \times g for 10 min. Four milliliters of the upper layer was transferred to a preweighed and precombusted glass vial and dried by nitrogen evaporation. The samples were dried for 2 h at 105°C, cooled in a desiccator, and weighed to determine the fat percentage. Milk sugar (lactose, glucose, and galactose) composition was determined by diluting 20 μ L of milk with 80 μ L of Milli-Q water followed by centrifugation at 21 000 \times g for 20 min at 4°C to separate fat and whey. Whey (60 μ L) was transferred to a clean tube and diluted ten times with Milli-Q water and further centrifuged at 16 100 \times g for 10 min at 4°C. Diluted whey (50 μ L) was separated isocratically by HPLC as previously described [18]. The total protein concentration of milk was determined using a Bradford assay [20] with α -casein (Sigma, Aldrich, Auckland; 70% α -casein, 30% other proteins) as the external standard.

2.2.2 SCFA analysis

Cecum digesta was collected post-mortem, snap frozen, and kept at –80°C prior to analyses. Cecum digesta (approximately 100 mg) was mixed with 1 mL of PBS and 30 μ L of internal standard (200 mM/L 2-ethyl butyric acid). Samples were homogenized using a hand-held homogenizer (OMNI international, Kennesaw, USA) at full speed for 10 s and then centrifuged (12 000 \times g for 5 min at 4°C) to remove particulate material, following which 1 mL of supernatant was transferred to a glass tube. Extraction of SCFA into an organic solvent and derivatisation were performed using a previously described method [21].

2.2.3 Leptin analysis

Blood was collected by cardiac puncture and serum leptin concentrations were measured using a Mouse Leptin Elisa kit (Millipore, Thermofisher NZ) according to the manufacturer's instructions.

2.2.4 Femur mineral composition

Both femurs were cleaned of all adherent tissues, weighed, then defatted and dried using two 16-h exposures to acetone and then ethyl ether following a previously published method [22]. Femurs were air dried and any visible remaining cartilage material was removed. Samples were placed in acid-leached, preweighed 20 mL borosilicate scintillation vials with acid-leached polypropylene caps and frozen at –80°C for 1 h. Femurs were then freeze dried overnight (FTS Systems, SP Scientific, Philadelphia, USA) and the dry weight recorded [23].

Bone samples were digested using a previously described method [24] with the following modifications. All samples received 2.5 mL HNO₃ (67% Aristar BDH, London, UK) and 0.5 mL HCl (34–37%, Trace Metal Grade Fisher) and heated in a foil covered aluminium block with acid leached funnels at 85°C for 1 h. Funnels were removed, and samples were heated up to 110°C until dry. Five milliliters of analytical matrix (i.e. 1 mL HNO₃ (67% Aristar BDH, London, UK); 1 mL HCl (34–37% Trace Metal Grade Fisher); 5 mL H₂O) were added and weighed, and samples decanted to acid-leached polypropylene 10 mL tubes for analysis.

Blanks were used to control for background levels in all steps (de-fatting, lyophilisation, and digestion). Samples were digested alongside blanks and Certified Reference Material (CRM) (IAEA-H-5) to test for contamination and to calculate recovery rates (Ca, 96–98%; Mg 93–100% and Zn 100%). Samples were measured on an Inductively Coupled Plasma MS instrument (ICP-MS, Thermo Electron Corporation, England) by Hill Laboratories, Hamilton, New Zealand, for calcium, magnesium and zinc concentrations (limits of detection: Ca 1.0 g/m³, Mg 0.4 g/m³, Zn 0.02 g/m³).

2.2.5 Colon histology and microbial analysis

Transverse sections of the proximal colon were collected and stored in 10% formalin solution at room temperature for subsequent assessment of colonic crypt length and goblet cell number.

In preparation for microbial analysis, approximately 50 mg of colon digesta DNA was extracted using a NucleoSpin Soil kit accordingly to the manufacturer's instructions (Macherey Nagel, Düren, Germany). For pyrotag sequencing, the V4-V6 region of the bacterial 16S rRNA gene was amplified. The primers and PCR conditions used have been previously published by our group [25]. Purified products

were pooled in equivalent quantities and sent to Macrogen (Seoul, Korea) for unidirectional sequencing from the forward primer using the Roche GS-FLX sequencer with Titanium chemistry. Sequences were analyzed accordingly with a method previously described [25].

2.2.6 PICRUSt analysis of 16S rRNA amplicon sequencing data

PICRUSt is a tool designed to infer metagenomics information from 16S rRNA amplicon sequencing data [26]. In the present study, analysis of 16S rRNA amplicon sequencing data was performed using the default settings of PICRUSt (version 0.9.1). Predicted enzymes/gene functions were grouped at level 2 of the KEGG Orthology and clusters of orthologous groups classification schemes for statistical analyses by single factor permutation ANOVA (500 permutations) as implemented by the RVAideMemoire package in R [27].

2.2.7 Statistical analysis

All data are presented as means with their standard errors. Statistical analysis was performed using ANOVA in GenStat version 12 (VSN International Ltd). Differences between means were considered significant when probability was less than 0.05 ($p < 0.05$). Trends were defined as $p > 0.05$ but < 0.10 .

Analysis of the colon microbiota sequencing data was performed using the nonparametric Kruskal–Wallis test in R 3.0.2 [28], the results of which were corrected for multiple testing using the Benjamini and Hochberg false discovery rate (FDR) adjustment.

3 Results

3.1 Body parameters

There was no evidence that diet affected dams' food intake (data not shown), body weight, GIT length, SI length or stomach, colon (no digesta), spleen, kidneys, brain, femur, and visceral fat weight (Supporting Information Table 2) during the experimental period. However, dams fed the CMO diet had greater colon length (control, 1.41 ± 0.04 ; GOS, 1.65 ± 0.14 ; CMO, 1.94 ± 0.14 ; mean [mm/g] \pm SE; p -value = 0.05) and lower liver weight (control, 85.8 ± 2.6 ; GOS, 86.0 ± 2.9 ; CMO, 73.1 ± 3.8 ; mean [mg/g] \pm SE; p -value = 0.05) than control fed dams.

At weaning, pups from dams fed the CMO diet had increased body weight (control, 4.69 ± 0.1 ; GOS, 4.98 ± 0.1 ; CMO, 5.20 ± 0.09 ; mean [mg/g] \pm SE; p -value = 0.02) and colon length (control, 3.06 ± 0.06 ; GOS, 3.33 ± 0.22 ; CMO, 4.52 ± 0.30 ; mean [mm/g] \pm SE; p -value < 0.001) compared to

pups from dams fed the control diet (Supporting Information Table 2). Body length was higher for weaned pups from dams fed the CMO and GOS diets (control, 5.17 ± 0.06 ; GOS, 5.35 ± 0.06 ; CMO, 5.38 ± 0.04 ; mean [mm/g] \pm SE; p -value = 0.04) than for pups from dams fed the control diet. After 30 days of receiving the control diet, pups from dams fed the CMO diet had increased visceral fat weight (control, 46.2 ± 1.7 ; GOS, 52.2 ± 2.5 ; CMO, 55.9 ± 2.8 ; mean [mg/g] \pm SE; p -value = 0.05) compared to pups from dams fed the control diet (Supporting Information Table 2).

3.2 Leptin serum concentration

Treatment did not affect dams' serum leptin concentration (control, 14.9 ± 1.1 ; GOS, 12.8 ± 2.1 ; CMO, 11.9 ± 1.8 ; ng/mL, mean \pm SE), however, 30 days after weaning, pups from dams fed the CMO diet had higher concentrations of serum leptin than pups from control-fed dams (control, 7.1 ± 0.8 ; GOS, 9.5 ± 0.9 ; CMO, 10.4 ± 1.2 ; ng/mL, mean \pm SE; $p = 0.05$).

3.3 Milk composition

Each milk sample utilized for compositional analysis is a pool of three milk samples collected from one animal at lactation days 5, 12, and 19. Due to variation in milk volume, three control; four GOS; and five CMO samples were analyzed for dry matter, total protein, and sugars and 1 control; three GOS; and two CMO samples were analyzed for fat content. Different sample numbers for total fat analysis ($n =$ Control, 1; GOS, 3; CMO, 2) a, b, Values with similar letters in rows do not differ significantly ($p < 0.05$). Feeding the CMO and GOS diets to the dams resulted in an increase of the total protein levels in milk (control, 74.8 ± 11.2 ; GOS, 115.9 ± 9.1 ; CMO, 115.0 ± 3.9 ; mg/mL, mean \pm SE, $p = 0.02$) (Supporting Information Table 3). Diet did not significantly affect dry matter, fat, lactose, glucose, or galactose concentrations in milk.

3.4 Femur mineral composition

There was no effect on femur mineral composition in dams consuming the CMO diet or their pups 30 days after weaning were observed (Supporting Information Table 4). At weaning, pups from dams fed the CMO and GOS diets had increased calcium concentration (control, 96.4 ± 2.7 ; GOS, 108.4 ± 2.9 ; CMO, 105.7 ± 2.0 ; mg/g, mean \pm SE, $p = 0.004$) in the femur compared to pups from dams fed the control diet. At weaning, pups from dams fed the GOS diet also had higher magnesium concentration (control, 2.58 ± 0.04 ; GOS, 2.75 ± 0.06 ; CMO, 2.62 ± 0.04 ; mg/g, mean \pm SE, $p = 0.02$) than pups from dams fed the CMO and control diets (Supporting Information Table 4).

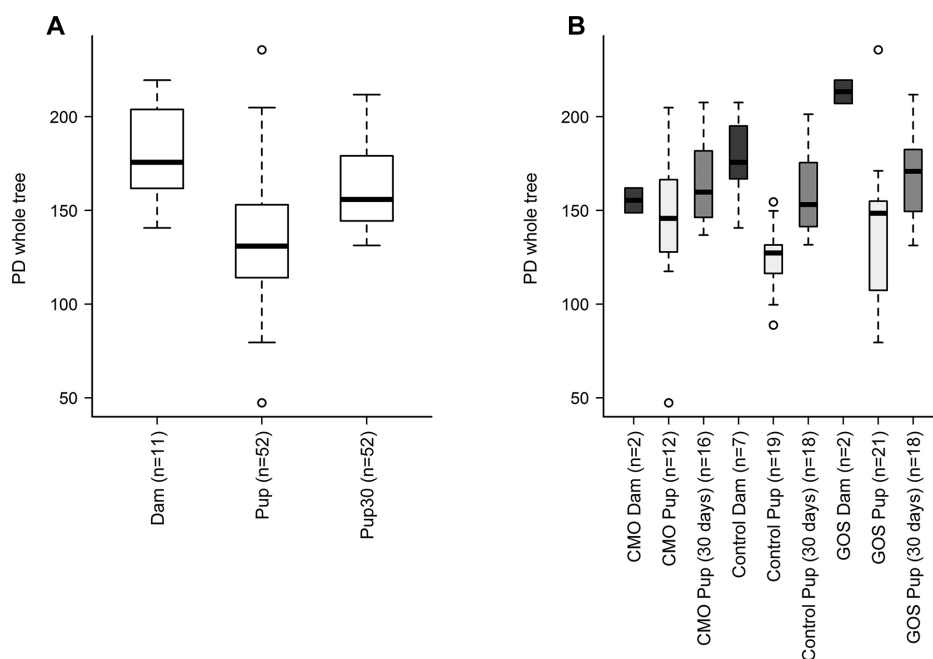


Figure 1. Boxplots of Faith's Phylogenetic Distances (2660 sampling depth) showing community diversity by (A) age and (B) age by diet. Significant differences in diversity were observed between dams, pups at weaning and pups 30 days postweaning ($*p < 0.001$). Communities from pups at weaning fed the CMO diet were also significantly more diverse than communities from pups at weaning fed the Control diet ($^{\#}p = 0.01$).

3.5 Cecum SCFA concentrations

CMO-fed dams showed higher concentrations of cecal formic acid than control diet-fed dams (control, 0.06 ± 0.02 ; GOS, 0.16 ± 0.05 ; CMO, 0.48 ± 0.17 ; $\mu\text{mol/g}$, mean \pm SE. p -value = 0.04) (Supporting Information Table 5). CMO and GOS-fed dams showed lower concentrations of cecal isobutyric acid than control-fed dams (control, 0.43 ± 0.02 ; GOS, 0.36 ± 0.03 ; CMO, 0.33 ± 0.02 ; $\mu\text{mol/g}$, mean \pm SE. $p = 0.04$). Dams fed the GOS diet showed higher concentrations of cecal propionic (control, 3.1 ± 0.1 ; GOS, 5.1 ± 0.3 ; CMO, 3.8 ± 0.3 ; $\mu\text{mol/g}$, mean \pm SE. $p = 0.01$) and butyric acids (control, 6.1 ± 1.0 ; GOS, 8.6 ± 0.5 ; CMO, 5.5 ± 0.7 ; $\mu\text{mol/g}$, mean \pm SE. $p = 0.04$) than dams fed the control or CMO diet.

Maternal dietary intervention was also shown to modify the pups SCFA production (Supporting Information Table 5). At weaning, pups from CMO-fed dams had a higher concentration of cecal butyric acid than pups from dams fed control or GOS diets (control, 0.7 ± 0.3 ; GOS, 1.3 ± 0.2 ; CMO, 2.9 ± 0.3 ; $\mu\text{mol/g}$, mean \pm SE. $p = 0.001$). Thirty days after weaning, pups from the dams fed the GOS diet showed higher concentrations of cecal propionic acid than pups from the dams fed the control diet (control, 1.3 ± 0.2 ; GOS, 2.9 ± 0.7 ; CMO, 2.4 ± 0.3 ; $\mu\text{mol/g}$, mean \pm SE. $p = 0.02$) (Supporting Information Table 5).

3.6 Colon histology and microbial composition

There was no evidence that diet influenced colon crypt length or goblet cell number of the dams or the pups (Supporting Information Table 6).

The microbiota composition of the colon contents of dams and pups after the experimental period was examined by high throughput 454 pyrosequencing of the bacterial 16S rRNA gene. Principal coordinate analysis (PCoA) of Unifrac phylogenetic distances showed that broad differences in community structure could be discerned between dams, pups at weaning and pups 30 days after weaning (Supporting Information Fig. 1A). However, differences between dietary treatments were less clear (Supporting Information Fig. 1B). Significant differences in community diversity were observed between pups at weaning, pups 30 days after weaning, and dams ($p < 0.001$) (Fig. 1A). Posthoc Wilcoxon rank sum analysis showed that the diversity of dams' microbiota was significantly higher than that of pups at weaning and 30 days postweaning, while the pups at 30 days postweaning had higher diversity than pups at weaning (Fig. 1A). Community diversity was significantly higher in pups at weaning fed the CMO diet compared to those fed the control diet ($p = 0.01$) (Fig. 1B). No other significant differences in diversity were observed between dietary treatments.

Although dietary intervention did not cause broad alterations in the microbiota of the dam colon digesta, the proportion of members of *Odoribacter spp.* (control, 0.4%; GOS, 0.6%; CMO, 1.8%) and *Oscillibacter spp.* (control, 0.7%; GOS, 0.1%; CMO, 1.5%) was increased in the colon digesta of CMO-fed dams compared to that of the GOS and control-fed dams (Supporting Information Table 7). Other members of the Firmicutes family, *Lactococcus spp.* (control, 0.02%; GOS, 0.14%; CMO, 0.05%), and the *Allobaculum spp.* (control, 0.3%; GOS, 3.9%; CMO, 1.9%) were found in higher proportions in the colon digesta of GOS-fed dams compared to that of the control and CMO-fed dams (Supporting Information Table 7).

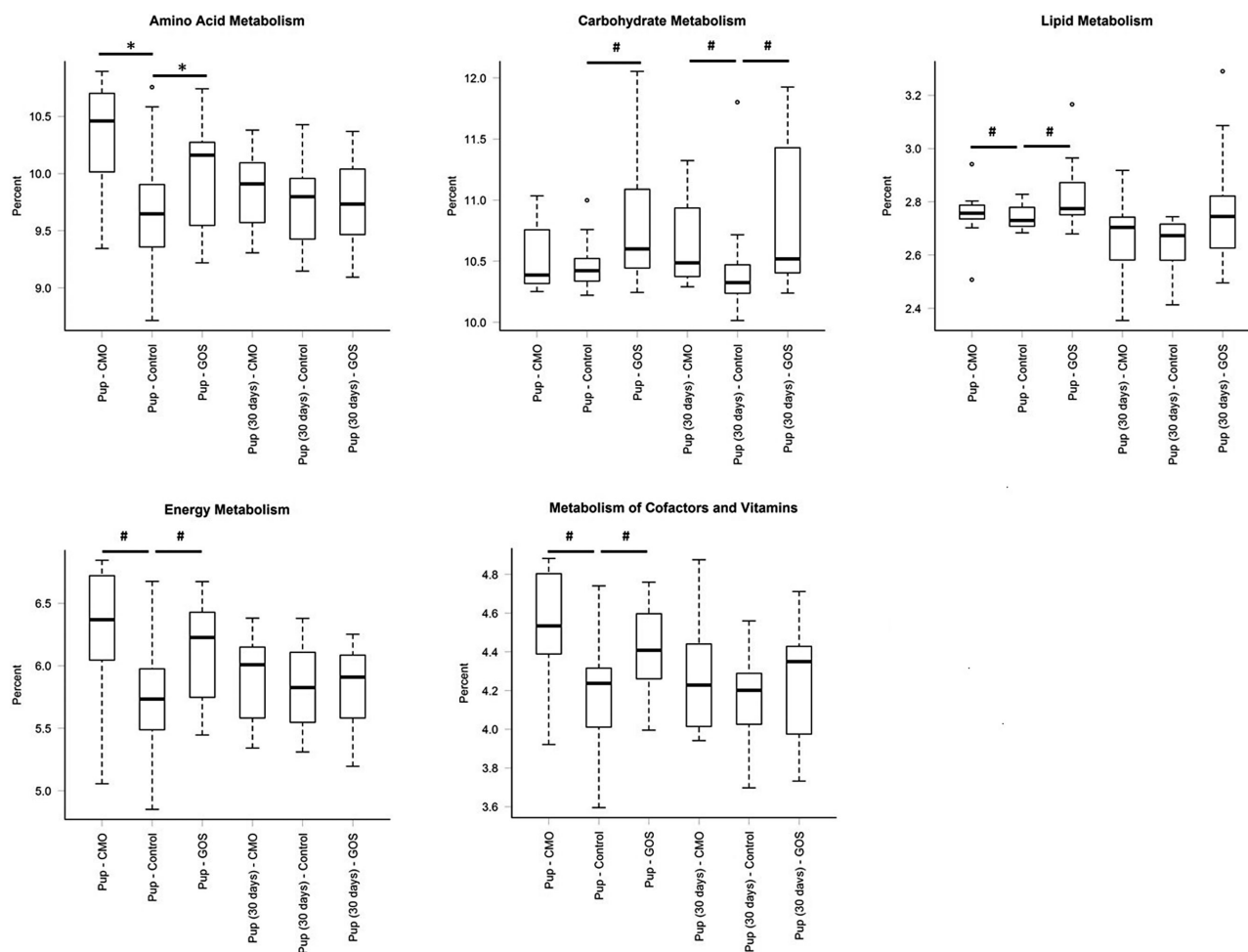


Figure 2. Relative abundances of predicted level 2 KEGG pathways involved in amino acid, carbohydrate, lipid, energy, and vitamin metabolism in the pups at weaning and 30 days postweaning compared within the groups (* $p < 0.005$, # $p < 0.01$).

At weaning, pups from dams fed the CMO diet showed genus alterations in the colon digesta community compared to pups from control and GOS-fed dams, with significantly higher proportions of *Bifidobacterium spp.* (control, 0.05%; GOS, 0.07%; CMO, 1.37%) and *Parabacteroides spp.* (control, 1.5%; GOS, 0.5%; CMO, 4.5%) (Supporting Information Table 7).

Thirty days after weaning, increased proportions of *Allobaculum spp.* (control, 5.5%; GOS, 3.0%; CMO, 10.7%) and decreased proportions of *Alistipes spp.* (control, 8.3%; GOS, 7.1%; CMO, 5.1%) were observed in pups from dams fed CMO diet compared to control. The greatest alterations in the colon digesta community were observed in pups from dams fed the GOS diet. Changes in the proportions of *Sporacetigenium spp.* (control, 0.2%; GOS, 1.9%; CMO, 0.5%), *Clostridium spp.* (control, 0.1%; GOS, 0.3%; CMO, 0.1%), *Turcibacter spp.* (control, 0.09%; GOS, 1.7%; CMO, 0.1%), unclassified Clostridiaceae (control, 0.1%; GOS, 0.3%; CMO, 0.08%) and unclassified Peptostreptococcaceae (control, 0.3%; GOS, 2.7%; CMO, 0.6%) were increased in pups

from dams fed GOS diet compared to pups from the dams fed the control and CMO diets, (Supporting Information Table 7).

3.7 Predicted metabolism-related gene functions in the colon microbiota associated with maternal diet

Overall the PICRUSt analysis showed a constant representation of metabolic functions across age and diet (Supporting Information Fig. 2). However, specific differences in the mean relative predicted abundance of gene categories (COGs and KOs) were found within age groups accordingly to the dam's diet (Fig. 2). Pups at weaning from CMO and GOS-fed dams had increased KEGG metabolic pathways of amino acid, energy, and vitamin metabolism compared to pups from control-fed dams. Pups at weaning from GOS-fed dams also had increased KEGG metabolic pathways of carbohydrate and lipids metabolism. Pups from CMO and GOS-fed dams had

Table 2. Summary of main findings on the effects of CMO and GOS diet compared to control diet on dams, pups at weaning, and pups 30 days postweaning. Similar letters in rows do not differ significantly ($p < 0.05$)

Animal	Parameters analyzed	Control	GOS	CMO		
Dam	Body parameters	Colon length	a	ab	↑b	
		Liver weight	a	a	↓b	
		Protein	a	↑b	↑b	
	Milk composition	Zinc	ab	↑a	b	
		Cecum SCFA				
	Main colonic taxa modulated by diet	Formic acid	a	ab	↑b	
		Propionic acid	a	↑b	a	
		Butyric acid	a	↑b	a	
		Isobutyric acid	a	↑b	↑b	
		Odoribacter spp	a	a	↑b	
		Oscillibacter spp	a	a	↑b	
	Pups weaning	Body parameters	Lactococcus spp	a	↑b	a
			Body weight	a	ab	↑b
Body length			a	↑b	↑b	
Femur mineral concentration		Colon length	a	a	↑b	
		Calcium	a	↑b	↑b	
		Magnesium	a	↑b	a	
		Zinc	ab	↑a	b	
Cecum SCFA		Butyric acid	a	a	↑b	
		Main colonic taxa modulated by diet				
Main colonic taxa modulated by diet		Bifidobacterim spp	a	a	↑b	
		Parabacteroides spp	a	a	↑b	
		Barnesiella spp	a	↓b	ab	
Pups 30 days postweaning	Body parameters	Visceral fat	a	ab	↑b	
		Serum leptin	a	ab	↑b	
	Cecum SCFA	Propionic acid	a	↑b	ab	
		Main colonic taxa modulated by diet				
	Main colonic taxa modulated by diet	Turicibacter spp	a	↑b	a	
		Sporacetigenium spp	a	↑b	ab	
		Clostridium spp	a	↑b	a	
		Alistipes spp	a	ab	↓b	
		Allobaculum spp	a	a	↑b	

↑ or ↓ indicate specific parameter increased or decreased compared to treatments with different letters. Number of animals used for the analysis of body parameters: dams (control, 8; GOS, 9; CMO, 7); pup at weaning (control, 27; GOS, 27; CMO, 23); pup 30 days postweaning (control, 18; GOS, 19; CMO, 16). Variations in the number of animals may have occurred for specific analysis, please see supplement material for exact number.

only an increase in carbohydrate KEEG metabolic pathways 30 days postweaning.

4 Discussion

The present study demonstrates that the consumption of CMO by dams during gestation and lactation is associated with changes in the colon microbiota, and in milk composition, which affected pups development. Our major findings, related directly or indirectly to CMO and GOS consumption compared to control diet are summarised in Table 2. The specific findings related to CMO consumption by the dams include (i) an increase in colon length in dams and pups at weaning; (ii) increased body weight and proportions of bifidobacteria in the colon of pups at weaning and (iii) increased body fat and serum leptin concentration in pups 30 days after weaning. The consumption of CMO and GOS by the dams also (iv) increased milk protein concentration; (v) altered cecal SCFA production in dams and pups at weaning; and finally (vi) increased body length and calcium con-

centration in the femur of pups at weaning. These findings highlight the potential for indirect exposures to nutrients via maternal diet to influence offspring development, GIT composition, and metabolism.

The CMO diet modified dams' colon microbiota by increasing the concentrations of the genera *Oscillibacter spp.* and *Odoribacter spp.* compared to dams fed GOS and control diets. Recent data suggest that the abundance of *Oscillibacter spp.* [29, 30] and *Odoribacter spp.* [31] are increased in obese individuals and negatively correlate with a functional intestinal barrier. Although, no difference was found in dams' body weight or visceral fat, a decrease of liver weight may indicate a decreased fat accumulation in this organ [32] and an altered lipid metabolism [33]. This is at odds with studies which have shown that the consumption of prebiotics by adults (human and rodents) and also pregnant mice improve the GIT microbiota composition, especially increasing the faecal concentrations of bifidobacteria and/or lactobacillus [8, 34].

The CMO diet also impacted cecal microbial fermentation through an increase in the concentration of formic acid

and a decrease in the concentration of isobutyric acid, compared to the control diet. Formic acid is a volatile SCFA rarely detected in nutritional studies [35], being an intermediate product, not an end-product, of bacterial fermentation which is converted readily to CO₂ and water. Another common end-product of bacterial fermentation, isobutyric acid, is produced by fermentation of proteins [36] when carbohydrate availability becomes a limiting factor for microbial fermentation [37]. Collectively, this could explain the decline in isobutyric acid in the cecum of CMO and GOS-fed dams, as the preferential metabolism of CMOF and GOS by carbohydrate-fermenting bacteria may have altered the microbiota and associated fermentation toward a type that was less proteolytic [38].

Increased milk protein concentration observed in dams fed the CMO and GOS diet may have contributed to increased body weight and length and increased muscle mass [11] observed in the pups at weaning. Accordingly, PICRUST analysis showed an increased representation of KEGG metabolic pathways of amino acid, carbohydrate, energy, and vitamin metabolism in pups at weaning from CMO and GOS-fed dams. However, apart from changes in carbohydrate metabolism, these differences in predicted functions were largely absent by 30 days postweaning. Early nutrition affects expression of genetic growth potential; this can have short-term and long-term effects on growth, development, metabolic programming, and disease risk [39]. In neonatal rats, higher protein intake via the enteral route was reported to enhance short-term weight gain, insulin resistance, and modification of the expression of glucose transporters. However, these differences were not sustained into adult life [40]. In human neonates, high protein milk formula (2 versus 1.5 g/100 mL) fed to babies considered small for gestational weight [41] resulted in an increase in total adiposity between 2 and 6 years of age [42]. The concentration of protein reported in murine milk, however, is highly variable (32 and 125 mg/mL) [19, 43], thus caution is needed to infer any physiological effect in the pups due to this variation.

As is typically observed as a consequence of direct high fiber intake [44], pups at weaning from CMO-fed dams had increased proportions of *Bifidobacterium spp.* and *Parabacteroides spp.* in the colon, and increased concentrations of butyric acid, in the cecum, as an indirect consequence of CMO intake by the dams. Increased in butyric acid in the dams cecum however, was only observed in dams which consumed GOS diet. The production of butyric acid is often positively correlated with the presence of bifidobacteria, most likely because bifidobacteria produce acetate and lactate, which are then converted by other bacteria (e.g., clostridial cluster 4, and 14a) into butyrate. Of the major SCFAs, butyrate is of special interest because it is the preferred energy source for colonocytes [45] and promotes a normal phenotype in these cells and hence may protect against cancer and other serious colonic diseases [46].

Dams' CMO intake also indirectly increased the microbial diversity in pups at weaning compared to pups from GOS and control-fed dams. Microbial diversity in the large intestine

in early life has been shown to be negatively correlated with the development of metabolic diseases and inflammatory bowel diseases later in life. It is certain, however, that limited direct exposure to the diets may have occurred when pups began to eat their dam's diet as they approached weaning [47]. Increased consumption of dietary fibres and production of butyric acid [48] are associated with increased intestinal length [49] and increased mineral absorption [50–53] as observed in this study by increased calcium concentration in the pups femur at weaning. The effects on colon length are usually accompanied by an increase in weight and crypt size [54–56] which was not observed in the present study. Changes in the milk oligosaccharide profile of the dam by diet (not determined in this study) may also favour the increase in the proportions of these commensal bacteria and increase colon length in the pups [57].

Thirty days after weaning, increases in the proportions of *Allobaculum spp.* and decreases of the genus *Alistipes spp.* were observed in pups from CMO-fed dams. *Allobaculum spp.* were reported to be affected by a high-fat diet [58] and dominant in the intestine of rodents fed a high fiber diet [59, 60] or prebiotic [58]. Conversely, 30 days after weaning, pups from dams fed the CMO diet had an increase in leptin concentration and fat mass without change in body weight. Interestingly, Desbouds et al., [61] reported a trend for increased leptin concentration 28 days after weaning in pups which had consumed the same prebiotic diet as the dams.

In conclusion, consumption of CMO or GOS by the dams during gestation and lactation differently altered dam's colon microbiota and cecal SCFA, although, both beneficially affected pup's development at weaning. Consumption of CMO diet only, however, increased the microbial diversity and the relative the abundance of bifidobacteria and butyric acid in the colon of pups at weaning. Specific changes of maternal GIT microbiota and liver weight may indicate that maternal lipid metabolism was modified by CMO dietary intake. Those effects could also be observed by an increase in body fat and leptin concentration in pups 30 days after weaning. Further studies, however, are needed to more comprehensively understand the physiological effects of CMO in the maternal/infant pair.

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