

# Perinatal caprine milk oligosaccharides consumption alters maternal and offspring liver gene expression

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## Research

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# Abstract

Consumption of caprine milk oligosaccharides (CMO) by dams during gestation and lactation, compared to a control diet or a diet supplemented with galacto-oligosaccharides (GOS), was associated with decreased maternal liver weight, increased offspring body weight and length at weaning, and increased offspring visceral fat and serum leptin concentration 30 days after weaning. These changes suggest that dietary CMO alters lipid metabolism, both in dams and offspring. We hypothesized that perinatal CMO intake affected expression of maternal genes in the liver involved in energy metabolism, and programmed pups' metabolic function leading to increased post-weaning visceral adiposity. To characterise the effects of perinatal consumption of CMOs on maternal and offspring liver gene expression, C57BL/6 mice were fed either a control, CMO, or GOS diet from mating to weaning. From weaning, half of the pups from each maternal group were fed the control diet for 30 days. Microarray analysis was conducted on liver samples from dams and offspring. Differences in the expression of genes involved in lipid metabolism were observed in dams and changes in expression of hepatic genes involved in energy balance *and* steroid metabolism were observed in pups at weaning. Increased visceral fat was observed in pups 30 days. Perinatal consumption of CMO diet affected infant lipid metabolism, which may be related to altered expression of genes in the liver involved in energy balance and lipid metabolism in dams.

## 1. Introduction

Maternal diet during pregnancy can affect both maternal health, and short and long-term health outcomes in the offspring (1). Human and rodent studies have shown that supplementing the maternal diet with prebiotics can improve maternal gut microbiota and offspring development by increasing weight gain, colon length, muscle and bone mass, and decreasing the incidence of allergies and asthma symptoms (2–5).

Caprine milk oligosaccharides (CMO) were shown to support the growth of gut beneficial bacteria in vitro studies (6, 7) revealing CMO had potentially had prebiotic effects. Using a mouse model, the consumption of CMO by dams during gestation and lactation, compared to the consumption of a control diet or diet supplemented with galacto-oligosaccharides (GOS), was associated with changes in the maternal colon microbiota, and in milk composition, which affected pup development (8). The specific findings related to CMO consumption by dams included (i) increased milk protein concentration; (ii) decreased liver weight in dams; (iii) increased colon length in dams and pups at weaning; (iv) increased body weight and length and proportions of *Bifidobacterium* in the colon of pups at weaning; and (v) increased body visceral fat and serum leptin concentration in pups 30 days after weaning. These findings highlight the potential for indirect effects of food components in the maternal diet on offspring development, gastrointestinal tract (GIT) composition, and metabolism.

Changes in colon microbiota and increased body fat and serum leptin concentration observed in dams and pups after maternal consumption of CMO may indicate that CMO altered lipid metabolism, both in dams and offspring. For example, 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 (8). Recent data suggest that the abundance of *Oscillibacter* spp (9, 10) and *Odoribacter* spp (11) are increased in obese individuals and correlate with decreased intestinal barrier function. Although no difference was found in dams' body weight or visceral fat, a decrease in liver weight may indicate decreased fat accumulation in this organ (12) and/or altered lipid metabolism (13). Altered lipid metabolism could also be observed in pups 30 days after weaning, which had an increase in visceral body fat and leptin serum concentration compared to pups from dams fed GOS or a control diet (8).

Rodent studies indicate that the consumption of prebiotics (e.g. inulin, oligofructose) has beneficial effects on lipid metabolism by positively altering an individual's serum lipid profile and decreasing hepatic steatosis (14). However, effects of perinatal prebiotic intake on the programming of offspring lipid metabolism have not been well established. We have previously reported the effects of CMO on liver adiposity and the gut microbiome (8). Here we describe liver gene expression in samples from the same study.

The hypothesis of this study was that perinatal CMO intake affected maternal liver genes involved in energy metabolism and programmed pups' metabolic function leading to increased post-weaning visceral adiposity. Therefore, the aim of this study was to determine the effects of perinatal consumption of CMO, GOS or a control diet on maternal and offspring liver gene expression.

## 2. Material And Methods

### 2.1. Animals and study design

As previously described (8), animal experimentation was approved by AgResearch Grasslands Animal Ethics Committee (Application No. 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, Hamilton, New Zealand and housed in a temperature and a 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 caprine milk oligosaccharide fraction (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). To ruled out the effect of GOS on maternal and offspring health outcomes 1% GOS was supplemented to the control diet.

CMOF was obtained by a previously described method (15). In short, caprine milk whey was processed by a combination of ultrafiltration, enzymatic hydrolysis of lactose, solid-phase extraction, and rotary vacuum evaporation. Oligosaccharide characterization and quantification were performed on a Thermo Scientific LTQ XL-Linear Ion TrapMass 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 ThermoHypercarb column (Thermo Fisher).

Mice were mated, and established pregnancy was evaluated by the presence of a vaginal plug. After delivery, pups remained with their dams up to weaning (21 days) where dams and half of the pups were euthanized by CO<sub>2</sub> and cervical dislocation and sampled. To determine the longer-term effects of maternal diet, the remaining pups were fed the control diet for 30 days, then euthanized. The left lobe of liver was collected from each animal and kept at -80°C until analysis. Complete experimental methods and results were previously described in Thum, et al (2016) (8).

## **2.2. Liver RNA extraction**

Gene expression profiles in the liver samples (n=4 animals per treatment per age group) was measured using Agilent Mouse Gene Expression 8x60K microarrays (catalog number G4852A; Agilent Technologies, California, USA), with each array consisting of 60,000 sequence probes, which map to 39,430 unique Entrez Gene identifiers, and 32974 unique gene symbols (16).

RNA and protein were extracted from liver tissue using the Qiagen AllPrep DNA/RNA/Protein Mini Kit according to the manufacturer's instructions (Qiagen Inc., California, USA). The RNA integrity was assessed using a RNA6000 Nano Labchip kit with an Agilent 2100 Bioanalyzer (Agilent Technologies), and only RNA samples with an RNA Integrity Number (RIN) greater than 6 used for subsequent analysis.

Extracted sample RNA and reference RNA were transcribed into cRNA and simultaneously labelled with cyanine dyes Cy3 or Cy5 according to the two-colour Quick Amp Labelling kit (Agilent Technologies). RNA that met quality criteria was transcribed into complementary RNA (cRNA) using T7 RNA polymerase and simultaneously amplified and labelled with the green fluorescent dye Cy3 or red fluorescent dye Cy5, in a dye-swap design, to avoid dye bias effect. The reference RNA used was extracted from intestine, kidney, liver and foetus of Swiss mice. The quality of the amplification and incorporation of the dye were assessed using a NanoDrop ND-1000 spectrophotometer, and only samples with a cRNA yield greater than 825 ng and a specific activity greater than 8 pmol Cy3 or Cy5 per µg cRNA were used. RNA Spike-in kits were included as instructed by the manufacturer (Agilent Technologies, California, USA).

Samples were hybridised onto Agilent Mouse Gene Expression 8x60K microarrays using four biological replicates per treatment/age, with 1 hybridisation for each biological replicate. Gene expression hybridisation kits were used as instructed. Microarray slides were placed in a hybridisation oven set to 65°C and 10 rpm for 17 h. Thereafter, slides were washed in Wash Buffer 1 and 2 (Agilent Technologies), acetonitrile, and Stabilisation and Drying solution (Agilent Technologies). Immediately after washing, slides were scanned at 5 µm resolution using an Agilent Technologies Scanner G2505B with feature extraction software (version 10.10.1.1) to extract the raw microarray images.

## **2.3. Microarray data analysis**

Microarray data were processed using the Bioconductor suite of packages in R (17). Differentially expressed genes were determined using an empirical Bayes modified t-statistic with the limma package (18). Genes with a >1.5-fold-change (FC) and a Benjamini and Hochberg false-discovery rate adjusted P-value ( $q$ ) < 0.05 were considered to be differentially expressed. Gene Ontology functions of the differentially expressed mapped genes were identified using the GeneMANIA (19) plugin for Cytoscape (20) using default settings. Additionally, gene set enrichment analysis (GSEA) was performed using the limma package in R on pathways listed in the Reactome curated database (21) (<http://www.reactome.org/>), with  $P < 0.05$  considered significant. It is important to note that, although validation of microarray data by qPCR is widely thought to be required, the reliability of using one technology to confirm the results of the other presents its own limitations. Nevertheless, it has been repeatedly demonstrated that the two methods yield comparable results, making repeated validation unnecessary. Furthermore, qPCR cannot be used to assess or confirm results from methods such as GSEA, which examine pathway-wide transcriptional changes.

### 3. Results

Dams fed the CMO, or GOS diet had 119 and 133 differentially expressed unique genes in the liver, respectively, compared to dams fed the control diet (Figure 1). The effects of maternal diet were also observed in the pups liver gene transcription where pups at weaning from dams fed CMO and GOS had 109 and 85 genes differently expressed, respectively. The longer-term effects of maternal diets were less prominent in pups; after they consumed the control diet for 30 days, only 7 and 2 liver genes were differently expressed in pups from dams fed the CMO and GOS diet, respectively (Figure 1).

We have previously shown that perinatal CMO intake did not affect maternal food intake nor body or visceral fat weight but lower liver weight (control,  $85.8 \pm 2.6$ ; GOS,  $86.0 \pm 2.9$ ; CMO,  $73.1 \pm 3.8$ ; mean [mg/g]  $\pm$  SE;  $p$ -value = 0.05) compared to control fed dams (8). Here we show that differentially expressed genes in dams fed the CMO diet compared to dams fed the control diet (Table 2, Supplementary material Figure 1) were primarily involved in the regulation of lipid metabolism or ubiquitination and SUMOylation (addition of a small ubiquitin-like modifier).

At weaning, pups from dams fed the CMO diet had increased body weight (control,  $4.69 \pm 0.1$ ; GOS,  $4.98 \pm 0.1$ ; CMO,  $5.20 \pm 0.09$ ; mean [mg/g]  $\pm$  SE;  $p$ -value = 0.02) and length (control,  $5.17 \pm 0.06$ ; GOS,  $5.35 \pm 0.06$ ; CMO,  $5.38 \pm 0.04$ ; mean [mm/g]  $\pm$  SE;  $p$ -value = 0.04) compared to pups from dams fed the control diet, as previously reported (8). Genes involved in the regulation of mitochondrial membrane permeability in apoptotic processes, cytochrome P450 *activity*, and steroid metabolism were differentially expressed in the liver of pups from dams fed CMO compared to pups from dams fed the control diet (Table 3, Supplementary material Figure 2).

After thirty days of receiving the control diet, pups from dams fed the CMO diet had increased visceral fat weight (control,  $46.2 \pm 1.7$ ; GOS,  $52.2 \pm 2.5$ ; CMO,  $55.9 \pm 2.8$ ; mean [mg/g]  $\pm$  SE;  $p$ -value = 0.05) and serum leptin (control,  $7.1 \pm 0.8$ ; GOS,  $9.5 \pm 0.9$ ; CMO,  $10.4 \pm 1.2$ ; ng/mL, mean  $\pm$  SE;  $p$  = 0.05) compared to pups

from dams fed the control diet (8). By thirty days after weaning only a small number of liver genes were differentially expressed in pups from dams fed CMO compared to pups from dams fed the control diet (Table 4, Supplementary material Figure 3), so Gene Ontology analysis using GeneMANIA was not possible. However, analysis of the pups' liver transcriptome by GSEA indicated other pathways were also affected by maternal diet in pups 30 days after weaning (Table 5). The expression of genes involved in the synthesis of epoxy (EET) and dihydroxyeicosatrienoic acids (DHET) was increased by 25%. Genes involved in collagen metabolism such as assembly of collagen fibrils (11%), collagen degradation (6%), collagen biosynthesis (6%) and formation (7%), and genes involved in extracellular matrix proteoglycans (6%) were down-regulated in pups 30 after weaning from dams fed the CMO diet compared to pups from dams fed the control diet (Table 5).

## 4. Discussion

### 4.1. Effect of CMO diet on maternal liver gene expression

This study showed that perinatal CMO intake affected expression of genes in the maternal liver related to energy and lipid metabolism compared to the control diet; changes included down-regulation of genes such as *Lcn2* (*lipocalin 2*) and *Prkag1* (*protein kinase AMP-activated non-catalytic subunit gamma*). Reduction of *Lcn2* (7.1 fold) and *Prkag1* (1.5 fold), a subunit of 5' AMP-activated protein kinase, has been linked to inhibition of hepatic fatty acid oxidation and promotion of cholesterol synthesis, lipogenesis, and triglyceride synthesis (22, 23). Concentrations of lipids and other metabolites, however, were not confirmed by metabolomic or histological analysis of the liver.

When the concentration of hepatic lipids increases, the formation of very low-density lipoprotein (VLDL) particles is stimulated for packaging and circulation of triglycerides, reducing lipid accumulation in the liver (24). Indeed, up-regulation of *Apob* gene expression (2.2 fold) was observed in the dams' liver; this gene provides instructions for making two versions of the apolipoprotein B protein, apolipoprotein B-48 and apolipoprotein B-100. Concentration of blood lipids, however, were not evaluated in this study. The time at which the dams' livers were sampled (weaning) could also affect *Apob* gene expression, as increased plasma triglyceride and apoB-48 concentrations in dams at weaning have been observed in rats (25). *Creb1* (cAMP-response element-binding protein), another up-regulated (1.6-fold) gene in dams fed CMO, has been shown to activate gluconeogenesis and fatty acid oxidation through increased expression of the nuclear hormone receptor peroxisome proliferator activated receptor gamma (PPAR- $\gamma$ ). However, PPAR- $\gamma$  expression was not changed in the liver of dams fed the CMO diet (26).

Genes involved in ubiquitination and SUMOylation were also among differentially expressed transcripts in the liver of dams fed CMO compared to the control diet (Table 2). Post-translational modification of target protein substrates by ubiquitin-like proteins (UbIs) and SUMO proteins regulates cellular signalling in numerous processes such as metabolism, transcription, translation, vesicle transport and apoptosis. Ubiquitination involves at least three classes of enzymes: ubiquitin-activating enzymes, or E1s, ubiquitin-conjugating enzymes, or E2s, and ubiquitin-protein ligases, or E3s (27). The genes *Ube2h*, *Ube2d3* and

*Ube2e3*, expression of which was higher in dams fed CMO, encode members of the E2 ubiquitin-conjugating enzyme family, while *Mul1* (mitochondrial ubiquitin protein ligase 1), *Rnf220* (*ring finger protein 220*), *Rnf111* and *Trim56* (Tripartite Motif Containing 56) genes encode for E3 ubiquitin ligases (Table 2). E3 ubiquitin ligases accept ubiquitin from an E2 ubiquitin-conjugating enzyme in the form of a thioester and then directly transfer the ubiquitin to targeted substrates. Among the different targets of E3 Ubs, *Mul1* has been shown to play a role in the control of mitochondrial morphology and to have anti-apoptotic activity while *Trim 56*, and both *Rnf111* and *Rnf220*, promote expression of a type I interferon IFN-beta in response to pathogens and enhance class I MHC mediated antigen processing and presentation. The expression of the SUMO specific peptidase 6 (*Semp6*) gene was increased in the liver of dams fed CMO compared to those fed the control diet; *Semp6* regulates genome stability, cell division, autoimmune responses and ageing (28).

Diet effects on the liver ubiquitome and its role in the regulation of whole-body euglycemia and lipidemia have recently been reported in rats (29). More specifically, it was observed that ubiquitination of proteins is a key regulatory mechanism controlling fatty acid metabolism which may mediate the pathogenesis of fatty acid-associated diseases. Variations observed in this study on liver ubiquitination genes and genes involved in lipid metabolism combined with reduced liver weight suggest that CMO may have modified liver lipid metabolism towards increased energy absorption and utilization.

#### **4.2. Effects of perinatal CMO consumption on offspring liver gene expression**

In contrast to our hypothesis, the effects of perinatal diet on pups metabolic function at weaning may have led towards energy expenditure and not accumulation. However, it is also important to note that changes observed in pups liver gene expression at weaning may also be influenced by maternal diet, as pups close to weaning may consume small amounts of maternal diet that may fall into the cage. Pups from dams fed the CMO diet, for example, had increased expression of two genes involved in steroid metabolism at weaning. The expression of both *Hsd3b5* (3 beta-hydroxysteroid dehydrogenase type 5) and *Srd5a1* (3-oxo-5 $\alpha$ -steroid 4-dehydrogenase 1) has been shown to negatively correlate with lipid accumulation in liver (30, 31). Although no changes in liver weight and other markers for liver lipid metabolism were found at weaning in pups from dams fed CMO, increased body growth (weight and length) may be correlated with changes in gene expression observed in the liver. Concentration of blood lipids, however, were not evaluated in this study.

Modulation of mitochondrial mass is also a major adaptive response to changes in energy balance, arising from decreases in oxygen or glucose availability, among other nutrient stresses (32). However, the effects of a maternal CMO diet on mitochondrial metabolism were not consistent, as the two pro-apoptotic genes differentially expressed in pups, Bcl-2 nineteen-kilo dalton interacting protein 3 (*Bnip3*) and Bcl-2-binding component 3 (*Bbc3*), were down-regulated and up-regulated respectively.

Other genes up-regulated in weaned pups from CMO-fed dams included those related to cytochrome P450s (CYP450) function. CYP450 monooxygenases are capable of catalyzing the metabolism of various endogenous and exogenous compounds, such as bile acids, fatty acids, retinoids, steroids, drugs

and other xenobiotics. The effects of diet on CYP450 have been established and reviewed (33). An increase in protein-to-carbohydrate ratio in the diet, for example, was shown to increase products of steroid hormone metabolism contributing to the transcriptional regulation of drug-metabolizing genes P450s (34). Indeed, weaned pups from dams fed the CMO diet had increased intake of protein-to-carbohydrate ratio, as maternal milk had higher concentrations of protein compared to control fed dams (8).

By thirty days after weaning, pups from dams fed CMO compared to control diet, had no indication of expected changes in liver functionality, previously linked with increased visceral fat weight and serum leptin concentration (35). Analysis of the pups' liver transcriptome by GSEA, however, showed that the expression of genes involved in the synthesis of epoxy (EET) and dihydroxyeicosatrienoic acids (DHET) and collagen metabolism were increased.

The EETs are signalling molecules formed within various types of cells by the metabolism of arachidonic acid by a specific subset of CYP450 enzymes. The EETs have been most studied in animal models where they show the ability to prevent arterial occlusive diseases such as heart attacks and brain strokes by their anti-hypertensive and anti-inflammatory effects on blood vessels (36). In the liver, collagens and proteoglycans play an intrinsic role in liver function in health and disease (37). The interaction of collagens and proteoglycans provide architectural elements for the liver with basement membrane or other duct architecture. They have mechanical roles like providing tensile strength and resilience, modulating diffusion and vascular flow, and regulating cell movement (37). Collagens and proteoglycans can also regulate signalling molecules such as growth factors, serving as ligands, storage depots and receptors, via multiple complex interactions between matrix proteins with other signal molecules or among different matrix components (38). However collagen deposition was not evaluated histologically in our study.

Traditionally, visceral obesity is strongly associated with insulin resistance, hypertension, dyslipidemia and systemic chronic low-grade inflammation, all of which play a pivotal role in the pathogenesis of atherosclerosis, thus increasing the risk of cardiovascular disease (39). However, as observed here, no expected changes on liver metabolism linked to the onset of metabolic diseases were observed in pups 30 days after weaning. This may be due to non-pathological residual effects of metabolic changes observed in early life or may be explained by mouse strain variations in response to diet-induced obesity (40).

## 5. Conclusions

Perinatal consumption of the CMO diet affected maternal and offspring liver gene expression, with effects most prominent in dams (119 genes) and pups at weaning (109 genes) compared to pups 30 days after weaning, which had consumed the control diet (7 genes). Differences in expression of ubiquitination and lipid metabolism-related genes in the dams' liver combined with previously reported reductions in liver weight and changes in colon microbiota (previously reported (8)) of the dams suggest

that CMO may have modified maternal lipid metabolism towards increased energy absorption and utilization. These effects may have been manifested in the offspring resulting in increased body growth (body length and weight) as well as increasing the expression of genes involved in energy balance *and* steroid metabolism. The previously reported increase in visceral fat mass, observed post-weaning, is unlikely to be explained by changes in pups liver gene expression (which were minimal), but instead could be a residual effect of metabolic changes observed in dams and pups at weaning triggered by maternal CMO intake. It is important to notice, there is not necessarily a straightforward correlation between gene expression and protein expression, protein activity (enzyme) and ultimately, metabolite(s) production by the liver. To deeper understanding of the effects of CMO on maternal and offspring energy metabolism, more studies are needed linking gene expression, metabolomic data and liver histology.

## 6. Declarations

Ethics approval

Animal experimentation was approved by AgResearch Grasslands Animal Ethics Committee (Application No. AE12579), in accordance with the New Zealand Animal Welfare Act 1999, New Zealand.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Author's contributions

C.T., N.R., and W.M. designed the study. C.T. supported by W.Y. performed the experiment, analyzed the data, and wrote the paper. All authors proof-read the paper.

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## Tables

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

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

1. <sup>a)</sup> 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. <sup>b)</sup> The sucrose concentration was adjusted in the CMO and GOS diets to balance the energy and nutritional content of the AIN-76A diet. <sup>c)</sup> Caprine milk oligosaccharides and their abundance: (13%) 3'- and/or 6'-galactosyl-lactose, (27%) 3'- and/or 6'-sialyl-lactose, (32%) 6'-glycolyl-neuraminyllactose, (9%) lacto-N-hexaose, (11%) disialyl-N-lactose, (8%) 6'-N-acetylglucosaminyllactose. Diet composition as previous described (8).

Table 2 Genes differently expressed in dams fed CMO compared to control diet.

<i>Pathway</i>	<i>GO ID</i>	<i>Gene ID</i>	<i>Gene name</i>	<i>Fold change in gene expression</i>	<i>FDR</i>
<i>Regulation of lipid metabolism</i>	<i>GO:0010878</i>	<i>NM_008491</i>	<i>Lcn2</i>	-7.11	0.001
		<i>NM_016781</i>	<i>Prkag1</i>	-1.5	0.04
	<i>GO:0010885</i>	<i>NM_009693</i>	<i>Apob</i>	2.23	0.03
		<i>NM_009952</i>	<i>Creb1</i>	1.62	0.01
<i>Ubiquitin-protein ligase and small ubiquitin-like modifier (SUMO) ligase activity</i>	<i>GO:0004842</i>	<i>NM_009459</i>	<i>Ube2h</i>	1.7	0.02
		<i>AK020443</i>	<i>Ube2d3</i>	1.7	0.04
	<i>GO:0019789</i>	<i>NM_010064</i>	<i>Ube2e3</i>	1.7	0.02
		<i>GO:0016881</i>	<i>NM_026689</i>	<i>Mul1</i>	-1.53
	<i>GO:0019787</i>	<i>NM_025739</i>	<i>Rnf220</i>	1.58	0.03
		<i>NM_033604</i>	<i>Rnf111</i>	2.3	0.04
		<i>NM_146003</i>	<i>Senp6</i>	1.7	0.001
		<i>NM_201373</i>	<i>Trim56</i>	1.7	0.02

GO ID: gene ontology identification number; FDR: false discovery rate.

**Table 3** Genes differently expressed in pups at weaning from dams fed CMO compared to control diet (n=4 per treatment per age group).

<i>Pathway</i>	<i>GO ID</i>	<i>Gene ID</i>	<i>Gene name</i>	<i>Fold change in gene expression</i>	<i>FDR</i>
<i>Regulation of mitochondrial membrane permeability involved in apoptotic process</i>	<i>GO:0097345</i>	<i>NM_009760</i>	<i>Bnip3</i>	-1.8	0.02
	<i>GO:1902110</i>				
	<i>GO:1902686</i>	<i>NM_133234</i>	<i>Bbc3</i>	2.63	0.02
	<i>GO:0035794</i>				
	<i>GO:1902108</i>				
<i>Cytochrome P 450 activity</i>	<i>GO:0004497</i>	<i>NM_009993</i>	<i>Cyp1a2</i>	3.45	0.02
		<i>NM_206537</i>	<i>Cyp2c54</i>	2.94	0.04
		<i>NM_001001446</i>	<i>Cyp2c44</i>	3.11	0.04
		<i>NM_007825</i>	<i>Cyp7b1</i>	3.38	0.04
		<i>NM_145434</i>	<i>Nr1d1</i>	2.09	0.008
<i>Steroid metabolism</i>	<i>GO:0016229</i>	<i>NM_008295</i>	<i>Hsd3b5</i>	3.97	0.04
		<i>NM_175283</i>	<i>Srd5a1</i>	3.03	0.04

GO ID: gene ontology identification number; FDR: false discovery rate

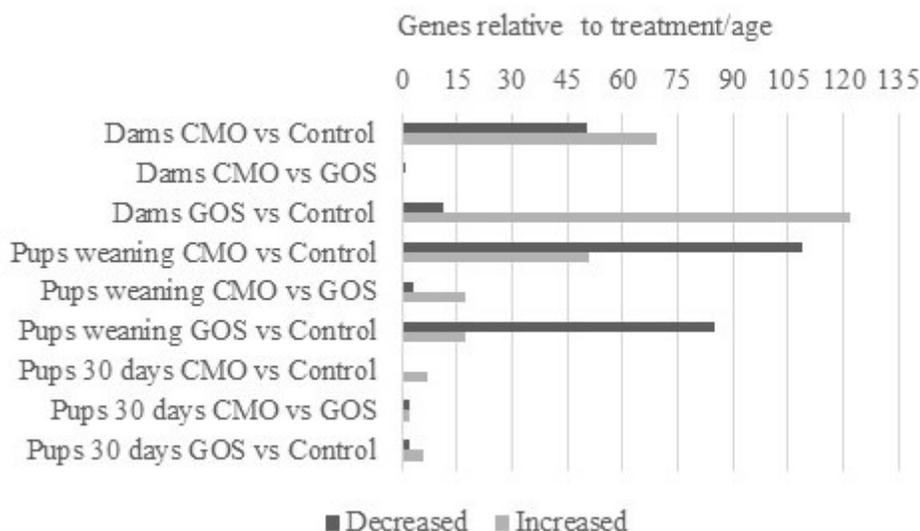
**Table 4** Genes differentially expressed in pups 30 days after weaning from dams fed CMO compared to pups from dams fed the control diet (n=4 per treatment per age group).

Gene ID	Gene name	Fold change in gene expression	FDR	Description
NM_001039562	Ankrd37	2.26	0.0005	Ankyrin repeat domain 37
NM_008871	Serpine1	2.31	0.005	Serine peptidase inhibitor.
NM_029568	Mfap4	1.90	0.02	Microfibrillar-associated protein 4
NM_011067	Per3	1.71	0.02	Period homolog 3
NM_008597	Mgp	3.09	0.02	Matrix Gla protein
NM_018874	Pnliprp1	2.42	0.03	Pancreatic lipase related protein 1
NM_028004	Ttn	5.46	0.04	Transcript variant N2-A

**Table 5** GSEA pathway analysis of genes significantly changed in the liver of pups 30 days after weaning from dams fed CMO diet (n=4 per treatment per age group).

Pathway	Number of Genes	Downregulated (%)	Upregulated (%)	P-value
<i>Synthesis of epoxy (EET) and dihydroxyeicosatrienoic acids (DHET)</i>	27	0	25	0.05
<i>Assembly of collagen fibrils and other multimeric structures</i>	54	11	5	0.02
<i>Collagen degradation</i>	92	6	1	0.03
<i>Collagen biosynthesis and modifying enzymes</i>	94	6	3	0.03
<i>Collagen formation</i>	100	7	3	0.04
<i>Extracellular matrix proteoglycans</i>	117	6	0	0.02

## Figures



**Figure 1**

Number of genes with a significant change in expression by each treatment/age (n=4 per treatment per age group).

## Supplementary Files

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