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Perinatal Caprine Milk Oligosaccharides Consumption: Altering the Maternal and Offspring Liver Gene Expression

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Research Article

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ABSTRACT

Background: 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.

Aim: This study aimed to characterise the effects of perinatal consumption of CMOs on maternal and offspring liver gene expression.

Methods: To liver gene expression, microarray analysis was conducted on liver samples from dams and offspring.

Results: 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. **Conclusion**: Perinatal consumption of CMO diet affects expression of genes in the liver involved in energy balance in dams, and in pups at weaning.

Keywords: Caprine milk oligosaccharides; Perinatal; Liver; Microarray; Lipid metabolism; Visceral fat

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INTRODUCTION

Caprine Milk Oligosaccharides (CMO) were shown to support the growth of gut beneficial bacteria *in vitro* studies [1,2] 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 [3]. 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 iv) 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. 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 [4] and/or altered lipid metabolism [5]. 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 [3]. Here we describe liver gene expression in the maternal and pup's samples from the same study.

MATERIALS AND METHODS

Animals and study design

As previously described [3], 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. In short, sixty-three C57BL/6 mice (42 female and 21 male) were obtained from the AgResearch Ruakura Small Animal Facility, Hamilton, New Zealand. Nine-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 [6].

After delivery, pups remained with their dams up to weaning (21 days) where dams and half of the pups were euthanized by CO_2 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) [3].

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

	Ingredients a	Control diet (AIN76A) in grams	GOS diet in grams	CMO diet in grams
Composition of diet	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	Control diet (AIN76A) in grams	GOS diet in grams	CMO diet in 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	1022.02	1033.6

Note: 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'-galactosyl-lactose and/or 6'-galactosyl-lactose, (27%) 3'-sialyl-lactose and/or 6'-sialyl-lactose, (32%) 6'-glycolyl-neuraminyl-lactose, (9%) lacto-N-hexaose, (11%) disialyl-N-lactose, (8%) 6'-N-acetyL-glucosaminyl-lactose; Diet composition as previous described.

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 8 × 60K 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 [7].

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 8 × 60K microarrays using four biological replicates per treatment/age, with 1 hybridisation for each biological replicate. Gene expression hybridisation kits were used as instructed by manufacturer (Agilent Technologies).

Microarray data analysis

Microarray data were processed using the Bioconductor suite of packages in R [8]. Differentially expressed genes were determined using an empirical Bayes modified t-statistic with the limma package [9]. 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 [10] plugin for Cytoscape [11] 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 [12] (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.

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).

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).

Figure 1. Number of genes with a significant change in expression by each treatment/age (n=4 per treatment per age group). **Note**: ■Decreased; ■ Increased.

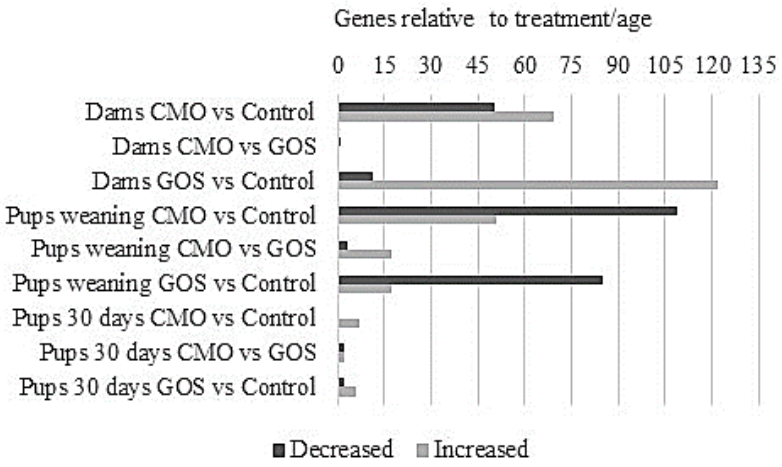

Table 2. Genes differently expressed in dams fed CMO compared to control diet.

Pathway	GO ID	Gene ID	Gene name	Fold change in gene expression	FDR
Regulation of lipid metabolism	GO:0010878 GO:0010885	NM_008491	Lcn2	-7.11	0.001
		NM_016781	Prkag1	-1.5	0.04
		NM_009693	Apob	2.23	0.03
		NM_009952	Creb1	1.62	0.01
Ubiquitin-protein ligase and small ubiquitin-like modifier (SUMO) ligase activity	GO:0004842 GO:0019789 GO:0016881 GO:0019787	NM_009459	Ube2h	1.7	0.02
		AK020443	Ube2d3	1.7	0.04
		NM_010064	Ube2e3	1.7	0.02
		NM_026689	Mul1	-1.53	0.009
		NM_025739	Rnf220	1.58	0.03
		NM_033604	Rnf111	2.3	0.04
		NM_146003	Senp6	1.7	0.001
		NM_201373	Trim56	1.7	0.02
Note: GO ID=gene ontology identification number; FDR=False discovery rate.					

At weaning, 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). 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). Analysis of the pups' liver transcriptome by GSEA (Table 5) indicated that the expression of genes involved in the synthesis of epoxy (EET) and dihydroxyeicosatrienoic acids (DHET) was increased by 25% and 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).

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

Pathway	GO ID	Gene ID	Gene name	Fold change in gene expression	FDR
Regulation of mitochondrial membrane permeability involved in apoptotic process	GO:0097345 GO:1902110 GO:1902686 GO:0035794 GO:1902108	NM_009760	Bnip3	-1.8	0.02
		NM_133234	Bbc3	2.63	0.02
Cytochrome P 450 activity	GO:0004497	NM_009993	Cyp1a2	3.45	0.02
		NM_206537	Cyp2c54	2.94	0.04
		NM_001001446	Cyp2c44	3.11	0.04
		NM_007825	Cyp7b1	3.38	0.04
		NM_145434	Nr1d1	2.09	0.008
Steroid metabolism	GO:0016229	NM_008295	Hsd3b5	3.97	0.04
		NM_175283	Srd5a1	3.03	0.04
Note: 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	1.9	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
Synthesis of epoxy (EET) and dihydroxyeicosatrienoic acids (DHET)	27	0	25	0.05
Assembly of collagen fibrils and other multimeric structures	54	11	5	0.02
Collagen degradation	92	6	1	0.03
Collagen biosynthesis and modifying enzymes	94	6	3	0.03
Collagen formation	100	7	3	0.04
Extracellular matrix proteoglycans	117	6	0	0.02

DISCUSSION

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-regulatation 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 [13,14]. Concentrations of lipids and other metabolites, however, were not confirmed by metabolomic or histological analysis of the liver.

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 (Ubls) and SUMO proteins regulates cellular signaling in numerous processes such as metabolism, transcription, translation, vesicle transport and apoptosis [15,16]. Diet effects on the liver ubiquitome and its role in the regulation of whole-body euglycemia and lipidemia have recently been reported in rats [17]. 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.

Effects of perinatal CMO consumption on offspring liver gene expression

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-5α-steroid 4-dehydrogenase 1) has been shown to negatively correlate with lipid accumulation in liver [18,19]. 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.

Other genes up-regulated in weaned pups from CMO-fed dams included those related to cytochrome P450s (CYP450) function. The effects of diet on CYP450 have been established and reviewed [20]. 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 [21]. 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 [3].

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 [22]. 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 signaling 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 [23]. In the liver, collagens and proteoglycans play an intrinsic role in liver function in health and disease [24]. The interaction of collagens and proteoglycans provide architectural elements for the liver with basement membrane or other duct architecture.

Traditionally, visceral obesity is strongly associated with metabolic diseases [25], 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 [26].

CONCLUSION

Perinatal consumption of the CMO diet affected maternal and offspring liver gene expression, with effects most prominent in dams and pups at weaning compared to pups 30 days after weaning, which had consumed the control diet. 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 of the dams suggest that CMO may have affected 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 trigged by maternal CMO intake. 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.

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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Authorship

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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