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# Does bacterial transformation of milk lipids occur in the infant bowel?

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## Executive Summary

We have preliminary data that show that particular bacterial species isolated from infant faeces chemically transform linoleic acid (LA) and docohexadecanoic acid (DHA) under controlled laboratory conditions. However, in real life the polyunsaturated fatty acids (FA) in milk may not reach the large bowel in significant amounts, or the bacterial species may be inactive with respect to fatty acid metabolism due to habitat restrictions. Confirmation of FA metabolites in infant faeces would support further *in vitro* laboratory-based studies to better understand the importance of dietary milk fats in infant nutrition and its relationship with bowel microbiota, leading to development of improved formulations for infant nutrition.

The aim of this project was to establish, whether or not fatty acid (FA) metabolites are present in the infant bowel. Faecal samples were collected from babies receiving different feeding regimes: Group 1: exclusively breast milk fed; Group 2: exclusively infant formula fed; and Group 3: mixed diet combination of breast milk and infant formula fed. Samples were collected from 44 babies across the three feeding regimes. To provide a measure of temporal consistency of variability within an individual, two samples were collected from 29 babies approximately 2-4 days apart, distributed across the three different groups. A total of 78 faecal samples were collected and analysed.

The presence and concentration of five polyunsaturated FA metabolites (1: *cis*-9,*trans*-11-conjugated linoleic acid *c*9,*t*11-CLA; 2: *trans*-9,*trans*-10-conjugated linoleic acid, *t*9,*t*11-CLA; 3: 10-hydroxy-cis-12-octacecenoic acid, HYA; 4: 10-oxo-12(Z)-octadecenoic acid, KetoA; and 5: 10-oxo-11(E)-octadecenoic acid, OxoA), as well other FA components, were determined. The average concentration of each metabolite associated with each feeding regime was calculated and statistically compared between groups.

**The main findings were:**

1. Microbial transformation of milk fat polyunsaturated fatty acids occurs in the infant bowel.
2. Variability in the faecal concentration of the metabolites was observed among individuals in the same group and also at different collection times for the same infant.
3. Milk diet does not appear to affect the faecal concentration of individual polyunsaturated fatty acids metabolites.
4. The presence of both CLA isomers was observed in all samples.
5. HYA was found in most of the samples. For samples collected from the same infant at two time points, HYA was not detected in one sample from the breast milk fed group, seven samples from the infant formula fed group and one sample from the mixed fed group.
6. OxoA was detected in samples but at levels below quantification limit. KetoA was not detected in any samples.

## Contents

# 1. Introduction

Fats are important components of infant nutrition, supplying 50 to 55% energy and helping the growth and development of the infant. Human milk and infant formula contain, saturated, monounsaturated and polyunsaturated fats that are important for regulating growth, inflammatory responses, immune function, vision, cognitive development and motor development systems in newborns.

Human milk contains a wide variety of lipid components, present as milk fat globules (MFG) with a core containing triglycerides (more than 98% of total lipids), surrounded by a MFG membrane (MFGM) with phospholipids, and esterified cholesterol and glycosylated lipids. Lipid composition of breast milk varies according to lactation stage, during the day and is strongly influenced by the mother's diet. In most infant formulas currently marketed, fat source used is a blend of vegetable oils, with a simpler composition than the lipid composition of breast milk, although the major fatty acids are similar in human milk and infant formula.

Recent studies have shown that bowel bacteria can metabolize polyunsaturated fatty acids, specifically linoleic acid, to generate several metabolites such as conjugated linoleic acid (CLA), hydroxyl fatty acids and oxo fatty acids that may affect host heath. Among the different metabolites produced by microbial transformation of linoleic acid, CLA has drawn significant attention since the 1980s. The biosynthesis *cis*-9, *trans*-11 CLA occurs initially through an isomerization process, where the double bond in the linoleic acid at carbon 12 position is transferred to carbon 11 position. The enzyme responsible for this transformation has been identified as linoleic acid isomerase (EC 5.3.1.5) and is found in bacterial cell membranes (Kepler et al, 1970). CLAs, such as *c*9,*t*11 CLA and *t*10,*c*12 CLA, reduce carcinogenesis, atherosclerosis and body fat. In terms of lipid metabolism, CLA is a potent peroxisome proliferator-activated receptor (PPAR)$\alpha$ agonist (Benjamin & Spener, 2009).

Based on these findings, CLA is commercialized as a functional ingredient for control of body weight (Benjamin & Spener, 2009). Because of its biological importance, CLA isomers have been measured in many types of dietary components, including infant formula and human milk. In human milk, *c*9,*t*11 CLA levels range from 2.23 to 5.43 mg/g fat, corresponding to 83 to 100 % of the total CLA present in human milk (McGuire et al, 1997). The isomer *t*10,*c*12-CLA is the second most abundant, corresponding to 0 to 20 % of the total CLA present (McGuire et al, 1997). In infant formula which contain vegetable oils as the main source of fat, CLA concentration is very low (McGuire et al, 1997).

The interests for other polyunsaturated metabolites, such as 10-hydroxy-cis-12-octacecenoic acid (HYA), 10-oxo-12(Z)-octadecenoic acid (KetoA) and 10-oxo-11(E)-octadecenoic acid (OxoA), is more recent. The metabolic pathways involved in the synthesis of these metabolites have been described by Kishino et al (2013). Functions of HYA include amelioration of intestinal epithelial barrier impairments by regulating TNFR2 expression (Miamoto et al, 2015) and anti-inflammatory effects *in vitro* in murine enterocytes (Bergamo et al, 2014). Feeding HYA to NC/Nga mice (a mouse model for atopic dermatitis) decreased the plasma IgE levels and skin infiltration of mast cells, with a concomitant decrease in the clinical dermatitis score (Kaikiri, et al, 2017). Other metabolites of linoleic acid may regulate energy metabolism of the host, e.g KetoA and OxoA activate PPAR$\gamma$ and stimulate adipogenesis (Goto, et al, 2015). There is no published information about the levels of HYA, KetoA or OxoA in the diet.

Altogether, these findings suggest that fatty acid metabolites produced by the microbiota have a health-enhancing potential for humans. Studies have demonstrated that the metabolites of linoleic acid, 10-hydroxy-*cis*-12-octadecenoic acid and 13-hydroxy-*cis*-9-octadecenoic acid, are higher in the intestine of pathogen-free mice compared with germ-free mice, demonstrating that gastrointestinal microbes play a role in modifying the FA profile of their host (Kishino et al, 2013).

Microbial colonisation of infant intestine is an intricate process which is influenced by many factors, including child's nutrition (breast milk or infant formula). Within the first year of life, the enteric microbiota is highly dynamic, and after the initial year, the microbial population stabilizes and resembles that of the adult. Different environmental factors during this critical period may influence the gut microbial composition, potentially impacting upon later life such as allergies and metabolic disorders (Chong et al, 2018, Koenig et al, 2011).

The aim of this study was to determine if the microbiota in the infant bowel has the capability to metabolize polyunsaturated FAs. We collected faecal samples from breast milk fed infants, formula-fed infants and mixed breast milk and formula fed infants and measured the levels of specific polyunsaturated FA metabolites in these samples.

# 2. Methodology

## 2.1 Samples

Samples were collected from babies at the Mothercraft, Waikato Hospital, Hamilton. Information related to infant gender, age, diet and ethnicity were recorded for each sample. All the data were anonymized and de-identified for study analysis. This study was approved by the Health and Disability Ethics Committees (16/STH/241).

Faecal samples were collected from babies receiving three different feeding regimes: Group 1: exclusively breast milk fed; Group 2: exclusively infant formula fed; and Group 3: mixed diet combination of breast milk and infant formula fed. Samples were collected from 44 babies across the three feeding regimes. To provide a measure of temporal consistency of variability within an individual, two samples were collected from 29 babies approximately 2-4 days apart, distributed across the three different groups. A total of 78 faecal samples were collected for analysis. After collection, samples were placed at -20$^{o}$C for up to one week and then stored at -80$^{o}$C until analysis.

## 2.2 Lipid extraction and methylation

Lipids were extracted using Folch protocol. Briefly, approximately 100 mg of faeces was mixed with water (0.5 mL), vortexed for 1 min, then 10 mL of methanol:chloroform (2:1) was added and the samples vortexed and placed in an orbital shaker for 3 hours at room temperature. The samples were, then centrifuged at 2000 rpm for 10 min. The liquid phase was transferred to a clean tube and the solvent was washed with 0.9% sodium chloride solution (2 mL). The solvent was evaporated and the lipid extract obtained was used in the derivatisation procedure.

To a heat resistant glass tube containing the lipid extract was added, 1.9 mg/mL heptadecanoic acid solution in hexane (50 μL) as internal standard and 1% sulfuric acid in methanol (1 mL). The tubes were tightly capped and incubated at 70°C for 1 hour. The samples were cooled to room temperature and 1 mL of sodium chloride saturated solution and 1 mL of hexane were added. The samples were vortexed for 1 min and centrifuged (1000 rpm) for 10 min. The top organic layer containing the fatty acid methyl esters (FAMEs) were transferred to a clean tube, and the hexane extraction was repeated. The organic phase were pooled, dried and re-dissolved in 200 μL hexane. The final solution was analysed using gas chromatography-mass spectrometry (GC-MS).

## 2.3 GC-MS

GC-MS analyses were carried out in a GC-MS QP2010 Shimadzu equipped with a fused-silica 2330 capillary column (100 m x 0.25 mm x 0.2 μM film thickness; Supelco). Oven temperature programming was 160°C isotherm for 2 min, increased to 175°C at 15°C/min, and isotherm for 15 min, and then increased to 250°C at 10°C/min isotherm for 25 min. The carrier gas (helium) flow at column was maintained at a constant velocity of 1 mL/min. The injector and interface temperatures were both set at 240°C. The MS instrument operated in positive ion mode using the standard 70 eV electron energy. Mass spectra were recorded in the *m/z* 43-500 range.

## 2.4 Data analysis

The structures of individual components were identified using the respective mass spectrum pattern fragmentation, as well as commercial standards. The quantification of the different fatty acids were estimated based on the area of the heptadecanoic acid used as internal standard.

## 2.5 Statistical analysis

ANOVA models were applied considering the main effects and their interactions. Multiple comparisons of treatments was performed by means of Tukey's honest significance test at 5% level of significance. Hierarchical cluster analysis (HCA) was applied to evaluate similarity among samples regarding concentration of fatty acids. The HCA was applied to data scaled to variance one (i.e. to eliminate the effect of different ranges of concentration among fatty acids) using Euclidean distance and Ward's minimum variance as clustering method. Data analysis was performed using R version 3.4.3.

# 3. Results and Discussion

A critical aim of this project was to monitor the presence of polyunsaturated fatty acids metabolites in infant faecal samples. Other studies have demonstrated that bowel microbiota is changed due to diets in earlier infancy which may have health implications (Koening et al, 2011). Data about the levels of polyunsaturated fatty acids metabolites will help us to gain a better understanding of the effect of the infant diet on the level of these metabolites.

## 3.1 Sample characteristics

Faecal samples were collected from 44 infants between the ages of 6-16 weeks (mean age 10.7 ± 3 weeks). Subject characteristics of our samples are shown in Table 1. At sample collection, 38.6% were exclusively breast milk fed, 36.3% were exclusively formula fed and 25% had mixed diet (breast milk fed and formula fed). The ethnicity of the infants that participated in the study were New Zealand European (81.8 %), Maori (6.8%), Asian (6.8%) and others (4.5%).

**Table 1.** Study characteristics

| **Characteristics** | |
|---|---|
| **Infant age at stool sample collection in week (SD)** | 10.7 (3.0) |
| **Infant gender n (%)** | |
| Male | 23 (52) |
| Female | 21 (48) |
| **Ethnicity n** | |
| NZ European | 36 |
| Asian | 3 |
| Maori | 3 |
| Others | 2 |
| **Feeding status n** | |
| Exclusive breast milk fed | 17 |
| Exclusive infant formula | 16 |
| Mixed breast milk fed and infant formula | 11 |

## 3.2 Concentration of polyunsaturated fatty acid microbial metabolites among all faecal samples

The average concentration (ng/mg) of *c*9,*t*11-CLA; t9,t11-CLA; and HYA in all faecal samples are presented in Table 2 and Figure 1. The most abundant metabolite was *c*9,*t*11-CLA for all feeding groups followed by *t*9,*t*11-CLA and HYA. OxoA was also detected in the samples but at a level below quantification limit, while KetoA was not detected. As *c*9,*t*11-CLA is the main CLA found in human milk and some infant formula (where the fat is not fully replaced by vegetable oils), its concentration in the faecal samples might be a combination of dietary and microbial metabolite. But for infant formula that vegetable oils are the main source of fatty acids CLA is not present as

dietary component. In this case linoleic acid present in the vegetable oil blends seems to be used by the gut microbiota for production of CLA.

Breast milk fed infants showed the lowest average concentration 376.4 ng/mg, 229.5 ng/mg and 69 ng/mg, of *c*9,*t*11 CLA, *t*9,*t*11 CLA and HYA, respectively. Infant formula fed infants show highest average concentration 592.9 ng/mg, 313.4 ng/mg and 154.8 ng/mg, of *c*9,*t*11 CLA, *t*9,*t*11 CLA and HYA, respectively. However, there was no statistically significant difference observed with concentration of metabolites and feeding regime, based on ANOVA and Tukey's honest significance test at 5% level of significance. Because this study measured faecal samples, lower amount of metabolites in the faeces could indicate a higher absorption of these metabolites by the infants. However, we cannot know with certainty as the food intake and milk composition were not recorded. Nonetheless, because these metabolites are associated with health benefits, it is positive to observe that the production of polyunsaturated microbial metabolites does not significantly change with diet status.

**Table 2.** Average of concentration of *c*9,*t*11-CLA; *t*9,*t*11-CLA; and HYA in faecal samples collected from infants fed different feeding regimes.

| Feeding | *c*9,*t*11-CLA (ng/mg) | *t*9,*t*11-CLA (ng/mg) | HYA (ng/mg) |
|---|---|---|---|
| **Breast milk fed** | 376.4 (116.9-618.5) | 229.5 (26.0-419.4) | 69.35 (5.64-128.8) |
| **Infant formula fed** | 592.9 (54.7-1131.2) | 313.4 (33.6 -593.4) | 154.8 (10.2-319.4) |
| **Mixed feed** | 548.26 (79.7-1016.8) | 416.2 (107.4-737.2) | 89.9 (33.9-145.9) |

*The values are presented as average and (interquartile range) in milligrams per gram of fresh faecal sample.* Statistical significant differences were not observed among concentration of metabolites and feeding regime based on ANOVA and Tukey's honest significance test at 5% level of significance.

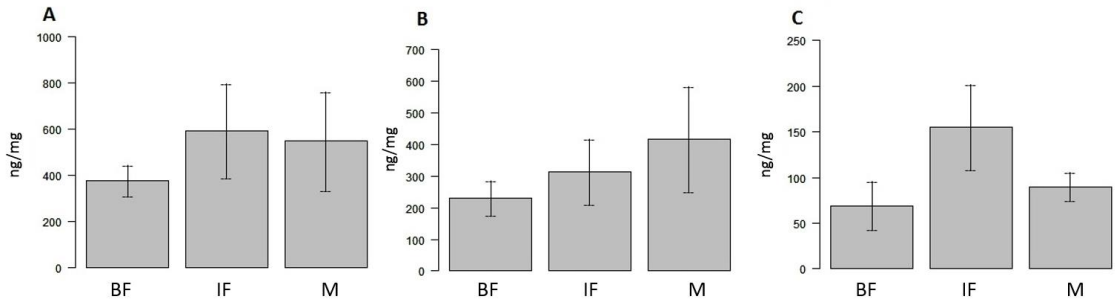


**Figure 1.** Faecal concentration of *c*9,*t*11-CLA (A), *t*9,*t*11-CLA (B) and HYA (C), according to the feeding status at fecal sample collection (BF – breast milk fed; IF – Infant formula fed; M – combination of breast milk fed and infant formula fed). Statistical significant differences were not observed among concentration of metabolites and feeding regime based on ANOVA and Tukey's honest significance test at 5% level of significance.

## 3.3 Concentration and temporal variation in the concentration of polyunsaturated fatty acids metabolites among dietary groups

Faecal samples of the same infant collected 1 to 4 days apart (Figure 2) were obtained from 29 infants corresponding to 58 samples. The results showed temporal variability. The temporal variability appeared to be higher in samples from breast milk fed and mixed fed infants than in formula fed infants. This may suggest that mother's nutrition and/or milk composition might affect the level of fatty acids in her breast milk. Fat in human milk is variable, changing in content over the course of a feed and during the day. Furthermore, FA composition itself can vary over the course of the day (Mitoulas et al, 2003, Daly et al, 1993). Infant formula fed babies would consume a constant fat diet. These factors may explain the greater variation observed in samples collected from babies being breast fed in this study.

CLA isomers were present in all samples. HYA was found in most of the samples, however, it was not detected in one sample of a breast milk fed group (one time point), one sample of mixed group (one time point) and in seven samples from infant formula fed group, which include both time points for 3 infants. Because this is the first time that these faecal metabolites concentration have been described, it was not possible to compare these results with previous studies.

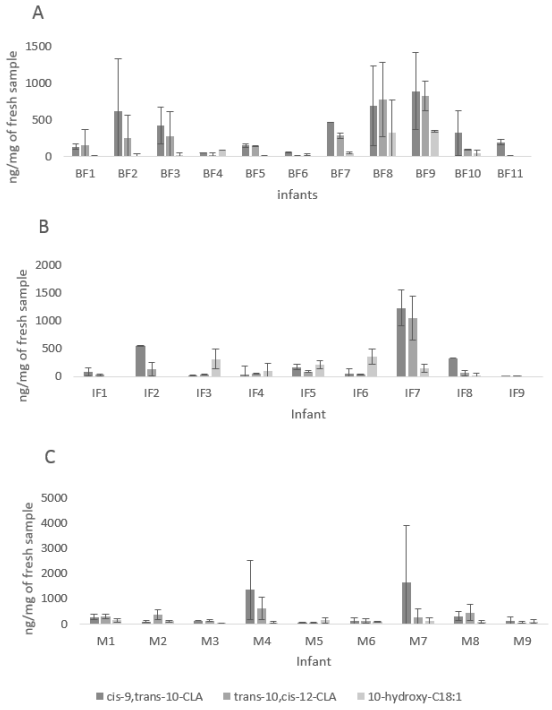


**Figure 2.** Concentration of *c*9,*t*11-CLA, *t*10,*c*12-CLA and HYA (10-hydroxy-C18:1) of 29 infants, according to the feeding status at fecal sample collection (A – breastfed; B – Infant formula fed; C – combination of breastfed and infant formula fed). The bars represent the standard deviation of the two samples of the same infant collected 1 to 4 days apart for the different infants.

## 3.4 Concentration and distribution of other fatty acids in all faecal samples

The concentration and distribution of 29 other fatty acids were measured in the faecal samples. The Hierarchical clustering graphic (Figure 3) shows that most of the breast milk fed (BF) infants are clustered in group 4, which show lower concentration of faecal fatty acids. Most of the infant formula (IF) fed infants are clustered in groups 3, 5 and 9 which show higher concentration of faecal fatty acids. No clear cluster was observed for mixed (M) fed infants; this group was distributed across all the different group clusters.

For preterm infants, studies have shown that the total fat absorption is not different between breast milk fed and formula fed infants at 6 weeks of age. However, the studies did show that the adsorption of specific fatty acid for some individual fatty acids were different between groups. The main difference was observed for long chain fatty acids, for example C22:6 (DHA) was absorbed less efficiently in formula fed compared with breast milk fed infants (74.9 vs 97.4, respectively) (Martin et al, 2016). In our study, we observed higher concentration of faecal fatty acids in infant formula fed infants, which may suggest lower absorption, however, the balance between intake and excretion was not performed.

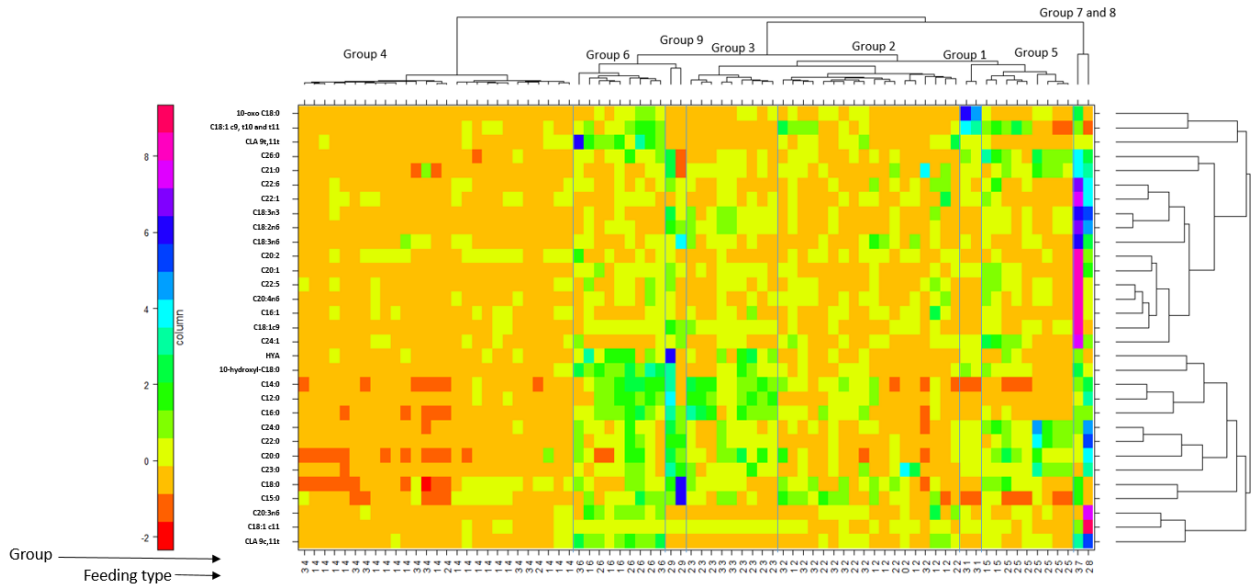


**Figure 3.** Hierarchical clustering applied to the concentration of all fatty acids identified in the different faecal samples (Group indicated the different clusters and feeding type 1 for BF; 2 for IF and 3 for M).

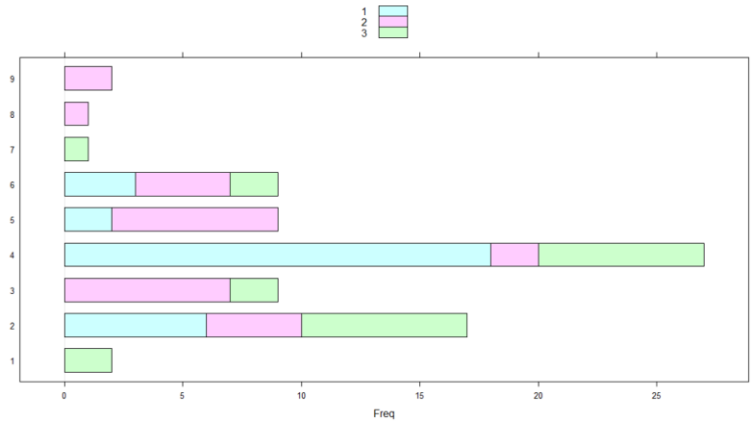


**Figure 4.** Number of infants per cluster group as per Fig 4. 1 – BF (blue); 2 – IF (pink); 3 – M (green).

## 4. Conclusion

This study shows for the first time that microbial transformation of milk polyunsaturated fatty acids occurs in the infant bowel. There was no statistically significant difference between faecal polyunsaturated fatty acids metabolites and infant diet (breast milk, infant formula and mixed diet), as large variations were observed among individuals in the same feeding group. This variability in the faecal concentration of these metabolites is worth further investigation as it might be linked to absorption rates among individuals and heath attributes.

However, in future studies we would recommend additional information is obtained, such as: composition of the mother diet and infant formula, as well health characteristics of the babies and mothers, including allergies information.

## 5. Acknowledgments

We gratefully acknowledge the staff of the Mothercraft at the Waikato Hospital (Wendy Diamond Hill, Jo Shea Kelly, Lorraine Reid, and Eleonor Carmichael), and Olivia Wallace (AgResearch) as well as the parents of our infants who were critical for the successful completion of this study.

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