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# Comparison of Short Chain Volatile Fatty Acids in the Breastmilk of Normal and Overweight/Obese Mothers

Ellen Gaskill

W Andrew Clark
*East Tennessee State University*

Kristy L. Thomas
*East Tennessee State University*

Amy Wahlquist
*Department of Biostatics and Epidemiology, East Tennessee State University*

Michelle E. Johnson
*East Tennessee State University*

# Comparison of Short Chain Volatile Fatty Acids in the Breastmilk of Normal and Overweight/Obese Mothers

Ellen R Gaskill

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|---|---|
| Ellen Gaskill<br>Ellen Gaskill, Author | 5/10/2022<br>Date |
| **W. Andrew Clark, PhD, RD, LDN**<br>Dr. W Andrew Clark, Faculty Mentor | **5/9/2022**<br>Date |
| Sean James Fox. PhD<br>Dr. Sean Fox, Faculty Reader | 5/9/2022<br>Date |
| *Michelle Johnson, PhD, RDN, LDN*<br>Dr. Michelle Johnson, Faculty Reader | **5/9/2022**<br>Date |

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**Abstract**

Health professionals emphasize the importance of breastfeeding in the development of children up to 6-months of age. It is known that short chain volatile fatty acids (SCVFAs) are a byproduct of nutrient fermentation by gut microbiome. These SCVFAs interact with the gut/brain axis and are known to influence infant development. Therefore, a reflection of maternal gut microbiome could likely be found in breastmilk (BM) due to diffusion of SCVFAs across the gut wall into the blood. Previous research in our laboratory has shown differences in the SCVFA fecal fermentation profile between individuals with normal (N) versus overweight/obese (OWOB) body mass index (BMI). Therefore, our research question is: Is there a difference in the relative amount and diversity of SCVFAs in the BM of N compared to OWOB women? We hypothesized that women of N will have a more diverse SCVFA profile than OWOB women in their BM. BM samples (200 ml) were collected from 44 women (22 N (BMI 22.0) and 22 OWOB (BMI 33.7) $p<0.0001$) between 40 and 65 days postpartum. Research participants read and signed an informed consent and were admitted to the study (IRB 0915.8sw-ETSU). N weight participants had a pre-gravid BMI between 18.5 and 24.9 $kg/m^2$ while OWOB participants had a pre-gravid BMI of greater than 25.0 $kg/m^2$. A 300 mg aliquot of lyophilized BM was placed in a separatory funnel with 5 ml of hexane and 5 ml of volatile fatty acid solution (VFA, oxalic acid (0.1M/L), sodium azide (40mM/L)). The funnel was rocked back and forth 50 times and placed on a ring stand to rest for 10 minutes. The bottom phase of the solution was collected and freeze-dried. Five hundred µL of VFA solution was added to the samples to resuspend, centrifuged (4,000 x rpm) for 20 minutes, the supernatant was removed and transferred to a microcentrifuge tube then centrifuged (12,000 x rpm) for 15 minutes and decanted. Three hundred µL of supernatant was transferred to autoinjector vials fitted with a 350 µL glass insert and analyzed

for SCVFAs via gas chromatography (GC) (Shimadzu) using a Phenomenex ZB-Wax Plus glass capillary column. SCVFAs acetate, propionate, isobutyrate, isovalerate and caproate were not different ($p>0.10$), while valerate ($p< 0.02$), isocaproate ($p<0.05$) and octanoate ($p<0.09$) were higher in the milk of N women. To our knowledge, this is the first time that SCVFAs have been quantified in the milk of lactating women using GC with an FID detector. This data supports the argument that the pre-gravid BMI of a mother can correlate to the SCVFA profile of her BM. It is unknown if the concentration observed in the mother's BM in this study has an influence on the neonate's gut/brain axis and neurological signals, however, we have demonstrated that the SCVFA profile is more diverse in the N BMI mother. Further research is warranted on the influence of maternal BM SCVFA composition on the growth and neurological development of her infant.

## Background

### BMI

Body mass index, or BMI, is a scale that is commonly used to determine if a person is within a healthy weight range for their height. The formula used to calculate BMI is the person's weight in kg divided by their height in meters, squared.[1] To be considered normal weight, one's BMI must be between 18.5 $kg/m^2$ and 24.9 $kg/m^2$.[1] If a person does not fall within the healthy range, they can fall into one of 4 other categories: severely underweight, underweight, overweight, or obese[1]. The main contributors to obesity and overweight are increased caloric intake and a sedentary lifestyle.[1] Being overweight or obese with increase a person's risk factor for many chronic diseases including coronary artery disease, nonalcoholic fatty liver disease, sleep apnea, type II diabetes, and more.[1]

### SCVFAS

Short chain volatile fatty acids (SCVFAs) are products of the digesting of carbohydrates and proteins in the gut.[2] They are defined as 1-7 carbon volatile fatty acids that can be found in the large intestine, and they can be either straight or branched-chain. SCVFAs can vary in distribution and therefore are associated with many different bodily processes being used as storage and signaling molecules as well as heavily influencing the gut microbiome.[3]

Acetic acid, propionic acid, and butyric acid can make up 90-95% of SCVFA in the gut, making them very influential to a person's health.[3] Increased propionate and butyrate are both related to reduced inflammation, especially in chronic diseases.[3] Acetate has also been shown to inhibit pathogens in the intestines and increase production of leptin which is known to reduce appetite.[3]

One study found that acetate can mediate joint inflammation as well.[4] Butyrate is a SCVFA that is known to alter the physiology of the colonic epithelium.[4] More specifically, it assists in regulation of tight junction proteins in the gut which enforces its integrity and prevents increased intestinal permeability which can lead to a number of inflammatory responses such as insulin resistance and obesity.[4] It is thought that acetate and butyrate can assist in lipid and glucose homeostasis.[4] Clearly, SCVFAs play a variety of roles in a mature gut, so research of them in other areas of the body could reveal vital health information.

**Breast Milk**

It is important to study SCVFAs in breastmilk because it has been shown that infants who are exposed to SCVFAs early in life can develop a more effective immune system later in life.[5] Various components of human breastmilk have been found to be protective for the infant, including acetate and butyrate.[6] These have been shown to improve immunological responses and to assist with beneficial growth and development.[6] BM is typically recommended by physicians because of benefits like this.

## Methods:

### Background

#### Participants

Forty-six Caucasian women were recruited from the BABE Breastfeeding Coalition of Tri-Cities support group on Facebook. These women donated approximately four ounces of milk between 45 and 60 days postpartum. Women who had or were being treated for mastitis and other breastfeeding disorders were excluded from the study. This study was separated into two groups: normal weight and overweight/obese. The requirement for the normal weight group was to have a BMI of between 18.5 $kg/m^2$ and 24.9 $kg/m^2$. To be in the overweight/obese category, the participant must have a BMI of 25.0 $kg/m^2$ or higher. Participation in this study was voluntary and the women in this study signed informed consent forms before donating their breastmilk.

#### Food Frequency Questionnaire

In order to understand what the mothers were taking in nutritionally we had each one take two food frequency questionnaires. The first questionnaire, Block Dietary Fruit-Vegetable-Fiber Screener, was ten questions long and was used to measure fruit/vegetable servings, vitamin C, magnesium, and dietary fiber intake. The second questionnaire was called the Block Dietary Fat Screener and it was used to measure their total fat, saturated fat, and dietary cholesterol intake along with percent fat and percent saturated fat. This questionnaire was seventeen questions. The equations (developed by Block et al.) used to calculate participants' scores are listed below.

Prediction equations for Block Dietary Fruit-Vegetable-Fiber Screener:

Fruit/Vegetable servings = -0.23 + [0.37 * (Fruit/vegetable score)] – (0.55 * Sex)

Vitamin C (mg) = 56.5 + [6.6 * (Fruit/Veg/Beans score)] – (26.7 * Sex) – (0.45 * Age)

Magnesium (mg) = 272 + [11.6 * (Fruit/Veg/Beans score)] – (92.3 * Sex) – (1.7 * Age)

Potassium (mg) = 2348 + [114.8 * (Fruit/Veg/Beans score)] – (759 * Sex) – (13.8 * Age)

Dietary fiber (gm) = 7.9 + [0.74 * (Fruit/Veg/Beans score)] – (4.5 * Sex)

Prediction equations for Block Dietary Fat Screener:

Total fat (gm) = 32.7 + [2.4 * (Meat/snack score)] + (11.2 * Sex)

Saturated fat (gm) = 9.4 + [0.88 * (Meat/snack score)] – (3.5 * Sex)

Percent fat (%) = 19.8 + [0.6 * (Meat/snack score)] + (2.3 * Sex)

Dietary cholesterol (gm) = 120 + [7.8 * (Meat/snack score)] – (54.65 * Sex) + (36.6 * Race)

## Self-Reported Demographics

Participants gave their current height and pre-gravid weight in order for their BMIs to be calculated. The equation used to find BMI was pre-gravid weight (kg) / [current height (m)]$^2$ = BMI. Participants also completed a 35-question demographic and health survey. This survey revealed information about their demographics, their health care services they used, their physical activity, their exposure to tobacco, their diet, their pregnancy, and their body weight.

## Laboratory Methods

### Breastmilk Sample Collection

Participants pumped approximately 4 ounces of milk using either an electronic pump or a hand pump between 45 and 60 days postpartum. Sample was kept refrigerated or frozen until donation and transported in a cooler to the Human Nutrition and Dietetics Research Lab at ETSU Valleybrook Campus. Samples were kept frozen at -30°C until freeze dried.

### Freeze Dry

Samples were placed in 600 mL LABCONCO flasks. The flask was placed on LABCONCO FreeZone 2.5 freeze dryer using stainless steel adapters. The samples ran for at least 24 hours on 0.077 mBar at -50 degrees Celsius.

### SCFA and LCFA Separation

Weighed out 0.300 grams of breastmilk (BM) onto weigh paper. Into a separatory funnel add 5 mL of hexane, 5 mL of volatile fatty acid solution (VFA, oxalic acid (0.1M/L), sodium azide (40mM/L)), and the 0.300 grams of BM. The flask was rocked back and forth 50 times to gently mix and set on ring stand for 10 minutes. This allowed the long chain fatty acids and other unwanted lipids to separate from the water-soluble short chain fatty acids. Once the 10 minutes were up, the bottom phase was drained into labeled 50 mL Falcon tubes. The top phase was discarded. The tubes were placed in a -80°C freezer until frozen (about an hour). These steps were run in duplicates for every sample.

### Freeze Dry

Four of the 50 mL Falcon tubes of frozen sample were placed in a 600 mL LABCONCO flask. Be sure there is airflow to every one of the Falcon tubes. The lid was put on, making

certain it is sealed. The flask was then placed on LABCONCO FreeZone 2.5 freeze dryer using stainless steel adapters. The samples ran for at least 24 hours on 0.077 mBar at -50°C. Samples were removed from the flask and the caps were replaced on the tubes. Repeated until every sample was freeze dried. Stored in a -80°C freezer until completely lyophilized.

Separation/Centrifugation

Samples were removed from the freezer. Five hundered µl of VFA solution was pipetted into each lyophilized sample to resuspend. The samples were vortexed to ensure they were fully homogenized and centrifuged at 4000 x rpm for 15 minutes. The supernatant was decanted into labeled microcentrifuge tubes and centrifuged at 12000 x rpm for 15 minutes. The supernatant was decanted and centrifuged again at 12000 x rpm for 15 minutes. Then, 300uL of the solution was pipetted into labeled amber autoinjector vials fitted with a 350µL glass insert. This process was repeated for all samples and stored in refrigerator until run in the gas chromatograph.

Gas Chromatography (GC)

All three gasses for the GC were turned on, making certain that there is at least 1/3 of gas left in the tank. Turn on the GC and the computer it is connected to. Then run on the GC with a Phenomenex ZB-Wax Plus glass capillary column. Collect and analyze the results.

Statistical analysis

Descriptive statistics including medians and interquartile range (25th to 75th percentiles) were calculated for outcomes of interest for each group (overweight/obese [OWOB] and normal weight[N]). Short chain fatty acid (SCFA) samples were run in duplicate, and values from the two runs were averaged together to create one composite score per individual. Wilcoxon ranked sum tests were used to compare SCFA outcomes between OWOB and N groups due to the skewed values and small samples sizes. Significance was determined at $\alpha = 0.05$ level.

**Results:**

**Table 1: Block Dietary Fruit-Vegetable-Fiber and Fat Screener**

| Item Measured | Normal Weight (Mean ± STD) (N=23) | Overweight/Obese (Mean ± STD) (N=23) | Significance (p<) |
|---|---|---|---|
| Fruit/Veg Servings per Day | 3.98 ± 1.62 | 4.75 ± 2.32 | (p<0.20) |
| Vitamin C (mg) | 137.55 ± 40.23 | 151.29 ± 58.90 | (p<0.36) |
| Magnesium (mg) | 342.87 ± 72.38 | 368.98 ± 105.75 | (p<0.33) |
| Potassium (mg) | 3290.98 ± 710.55 | 3542.88 ± 1038.74 | (p<0.34) |
| Dietary Fiber (mg) | 16.95 ± 4.90 | 18.79 ± 7.15 | (p<0.31) |
| Total Fat Score | 26.13 ± 6.40 | 26.00 ± 6.73 | (p<0.95) |
| Total Fat (gm) | 106.60 ± 15.35 | 106.30 ± 16.14 | (p<0.95) |
| Saturated Fat (gm) | 28.89 ± 5.63 | 28.78 ± 5.92 | (p<0.95) |
| Percent Fat | 37.78 ± 3.84 | 37.70 ± 4.04 | (p<0.95) |
| Dietary Cholesterol (mg) | 269.12 ± 49.88 | 273.14 ± 58.60 | (p<0.80) |
| Percent Saturated Fat | 10.21 ± 1.58 | 10.18 ± 1.67 | (p<0.94) |
| Pre-gravid BMI | 22.03 ± 1.83 | 33.67 ± 6.14 | (p<0.0001) |
| BMI (at 18 years old) | 20.46 ± 2.62 | 27.77 ± 5.71 | (p<0.0001) |
| BMI (Highest) | 23.39 ± 2.26 | 35.60 ±6.21 | (p<0.0001) |

*Table 1:* No statistical significance was found between the two groups in regards to their nutrient intake. The BMI of the two groups was statistically different before their pregnancy ($P<0.0001$), at their highest BMI during pregnancy ($p<0.0001$), and from when they were 18 years old ($p<0.0001$).

Table 2: Moderate-Intensity Physical Activity

| Item Measured | N Weight (Mean ± SD) | OWOB (Mean ± SD) | Significance (p<) |
|---|---|---|---|
| Days per week of moderate-intensity activity | 3.38 ± 2.45 | 2.57 ± 2.22 | p=0.276 |
| Hours per day of moderate-intensity activity | 2.02 ±3.02 | 2.51 ± 3.41 | p=0.618 |
| Days per week of moderate-intensity fitness or sport | 1.17 ± 1.65 | 1.55 ± 1.65 | p= 0.451 |
| Hours per day of moderate-intensity fitness or sport | 0.26 ± 0.37 | 0.53 ± 0.68 | p=0.101 |

*Table 2:* There was no statistical significance found in the physical activity of the mothers between the two groups.

Table 3: Prenatal Vitamin Consumption during Pregnancy and Lactation

| Item Measured | N Weight | | OWOB | |
|---|---|---|---|---|
| | Yes | No | Yes | No |
| Took prenatal vitamins during pregnancy | 24 | 0 | 19 | 1 |
| Took prenatal vitamins at time the breast milk was collected | 15 | 9 | 18 | 2 |

*Table 3:* Twenty-four of the N weight women took prenatal vitamins during pregnancy and 15 of them took prenatal vitamins postpartum. Nineteen of the OWOB women took prenatal vitamins during pregnancy and 18 continued taking them postpartum.

Table 4: Dietary Information

| Item Measured | N Weight | | OWOB | |
|---|---|---|---|---|
| | Yes | No | Yes | No |
| Take a daily fish oil/ krill oil supplement | 6 | 18 | 3 | 17 |
| Routinely eat fish | 11 | 13 | 6 | 14 |
| Eat nuts | 21 | 3 | 19 | 1 |
| Eat flaxseed or take flaxseed oil | 6 | 18 | 7 | 13 |
| Take dietary supplements (other than prenatal vitamin) | 7 | 17 | 4 | 16 |

*Table 4:* More of the N weight women took each of the supplements they were asked about except flaxseed/flaxseed oil. More OWOB women took these supplements than N wright women.

**Table 5: SCVFA Area Percent**

| | Normal Weight (N=23) | | | Overweight/Obese (N=23) | | | |
|---|---|---|---|---|---|---|---|
| | Median | 25th Percentile | 75th Percentile | Median | 25th Percentile | 75th Percentile | p-value |
| Acetic | 33.55 | 26.46 | 50.13 | 43.95 | 32.45 | 54.56 | 0.22 |
| Propionic | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.45 |
| Isobutyric | 0.00 | 0.00 | 1.46 | 0.86 | 0.00 | 3.24 | 0.18 |
| Butyric | 23.23 | 12.09 | 32.22 | 17.63 | 12.06 | 25.56 | 0.11 |
| Isovaleric | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.81 |
| Valeric | 0.00 | 0.00 | 2.54 | 0.00 | 0.00 | 0.00 | 0.02 |
| Isocaproic | 0.00 | 0.00 | 0.61 | 0.00 | 0.00 | 0.00 | 0.05 |
| Caproic | 26.94 | 21.79 | 30.18 | 24.12 | 14.79 | 27.71 | 0.28 |
| Octanoic | 6.08 | 2.40 | 9.65 | 9.27 | 4.30 | 12.08 | 0.09 |

*Table 1*: Three of the nine SCVFAs that were analyzed had statistical difference in area percent between the N and OWOB groups. These were valeric ($p<0.02$), isocaproic (0.05), and octanoic (0.09). Butyric was approaching statistical significance at $p<0.11$. The N group had higher area percent in butyric, valeric, and isocaproic, while the OWOB group had higher area percent of octanoic.

**Discussion:**

The women of the normal weight group consumed slightly more total fat, saturated fat, percent fat, and percent saturated fat than the OWOB group. On the other hand, the OWOB group consumed more vitamin c ($p<0.36$), magnesium ($p<0.33$), dietary fiber ($p<0.31$), dietary

cholesterol ($p<0.80$), and more fruit and vegetable servings per day ($p<0.20$). Although there were numerical differences, none of these were statistically significant.

There was no statistical significance found in the physical activity questionnaire, however the OWOB mothers had slightly more days and hours of physical activity($p>0.10$) as well as fitness and sport ($p>0.10$) than the normal weight mothers in all the categories.

More of the N weight women took prenatal vitamins during pregnancy than OWOB women, however, more of the OWOB women continued to take them postpartum. More normal weight women took fish oil or krill oil supplement, routinely ate fish, ate nuts, and took supplements other than prenatal vitamins. More of the OWOB women consume flaxseed or flaxseed oil. No significance was found between normal weight and OWOB for any of these groups.

The only significance found between the two groups when it comes to area percent of SCVFA is valeric ($p<0.02$), isocaproic ($p<0.05$), and octanoic ($p<0.09$). For valeric and caproic, there is a higher area percent in the normal weight mothers, while octanoic has a higher area percent in the OWOB mothers. Butyric ($p<0.11$) had a higher area percent in normal weight mothers and was approaching significance. Caproic ($p<0.28$), although not significant, also had a higher area percent for the normal weight mothers. Acetic and isobutyric were not significantly different ($p>0.10$) but were numerically higher in the OWOB group.

## Conclusion:

We hypothesized that the BM of N mothers would have a higher concentration and higher variability of SCVFAs than OWOB mothers. This was supported by our data in that valeric ($p<0.02$), isocaproic ($p<0.05$), and octanoic ($p<0.09$) were all significantly different

between groups. There was a higher area percent in these SCVFAs in the N weight mothers than in the OWOB mothers. All in all, our hypothesis is supported by this. From here a study could be done to see how this could affect growth and development of the child. There is also and how it could affect the gut-brain axis of the child.

There was no statistical difference found in the activity levels or the food intake between the two mothers. There was a significant difference between the groups' BMIs both before ($p<0.0001$) and during ($p<0.0001$) pregnancy. Although the mothers didn't have statistical differences between their intake and their activity levels, the two groups did have statistically different BMIs at age 18 ($p<0.0001$).

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## Appendix


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