BRANCHED CHAIN FATTY ACIDS IN THE NEONATAL GUT, AND ESTIMATED PREVALENCE IN THE AMERICAN FOOD SUPPLY

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Rinat Rivka Ran-Ressler

January 2011

BRANCHED CHAIN FATTY ACIDS IN THE NEONATAL GUT, AND ESTIMATED PREVALENCE IN THE AMERICAN FOOD SUPPLY

Rinat Rivka Ran-Ressler, Ph. D.

Cornell University 2011

Branched chain fatty acids (BCFA) are found at trace levels in most non-ruminants, but are major components of vernix. Vernix suspended in amniotic fluid is normally swallowed by the fetus, increasingly so as parturition approaches. The first study investigated whether BCFA are a component of the newborn gut by measuring BCFA distribution in vernix and meconium of newborns. Results indicated that BCFA are normal constituents of the term newborn gastrointestinal (GI) tract. Furthermore, differences found in the BCFA distribution between vernix and meconium suggest that the fetal gut metabolizes BCFA.

Given that BCFA are constituents of the newborn GI tract, the second study examined whether lack of GI exposure to BCFA, due to premature birth, is associated with the risk for necrotizing enterocolitis (NEC). Rat pups fed rat milk formula with BCFA had less NEC compared to pups fed a standard formula. A three-fold increase in the ileal anti-inflammatory, IL-10, was also detected in the BCFA-fed group. BCFA were selectively incorporated into ileum phospholipids. These results indicate that BCFA have a protective effect against NEC and that this effect may be due to increased ileal anti-inflammatory cytokine levels. This suggests that BCFA are metabolized in the ileum, where they may be associated with the reduction in NEC.

As data on possible beneficial roles of BCFA emerge, their presence in the US food chain becomes valuable. The third study examined BCFA distribution in retail milk

a major contributor to energy intake among most segments of the population. BCFA were found to comprise 2% of the total FA in retail milkfat and estimated intake suggests that BCFA can constitute a substantial amount of daily fat intake.

These studies indicate that BCFA are normally present in the human gut throughout the life cycle and may be beneficial to human digestive health. They are native to the American diet and appear to help maintain perinatal gut health. These results invite continued consideration of the health effects of BCFA in human nutrition, and particularly inclusion in preterm infant formula. Their estimated intake in the American diet suggests that future studies explore their possible health benefits.

BIOGRAPHICAL SKETCH

Rinat Rivka Ran-Ressler was born in Holon, Israel, to Ahron and Ofra Ran, a family eight generations in Israel. After serving in the Israeli Defense Forces as an officer, Rinat started her Bachelor of Science degree in Nutritional Sciences in the Agricultural Faculty of the Hebrew University in Rehovot, Israel. After graduation, Rinat did her dietetic internship at Hadassah Ein-Kerem Hospital in Jerusalem, and upon her completion of the internship stayed to work as a registered dietician in Hadassah Hospital for the next 6 years. During this time Rinat was featured in nutrition segments on the morning television show "*Boker Tov Yisrael*" ("Good Morning, Israel") and earned her MBA at Israel's Ben-Gurion University, in Beer Sheva, where she met her husband, Dr. William Harris Ressler. After graduation, Rinat became the Scientific Manager of Materna Laboratories, Ltd, an infant formula manufacturer in Israel. During this time, her children Tomer and Noa were born. During seven happy years in Materna Laboratories, Rinat developed a passion for research; she applied to Cornell University and upon acceptance moved with her family to Ithaca, NY, and began her Ph.D. studies under Professor J. Thomas Brenna. Rinat completed the requirements for her Ph.D. in February 2011 and stayed as a post–doctoral associate in Dr. Brenna's laboratory.

To my late sister Ravit, the wind beneath my wings

לרוית, הרוח במפרשיי

ACKNOWLEDGMENTS

Five years ago, at the new graduate student orientation in the DNS, I introduced myself and said that I came to Cornell from Israel to get my Ph.D. in lipids in infant nutrition. I would like to thank you, Tom, for making it possible for me to do my dissertation on what has turned out to be such an interesting and novel subject. Thank you for sharing your wealth of knowledge in nutrition in general and in lipids and fatty acids in infant nutrition in particular. Thank you for your mentorship and for supporting me in almost every possible aspect of learning how to carry out good research.

I would like to thank a number of other people, who have been so important in my life these past five years and without whom I never would have completed this dissertation.

To my committee members: Dr. Liu, for his advice on working in industry and for always being interested in my work, Dr. Weiland, for opening before me a molecular world, in which I had thought I had not interest, but which turned out to be especially engaging, and Dr. Utermohlen, for the wise advices and the inspiring conversations.

To Pete, for being such a central source of information and support, for showing me the basic steps in lab work in general and in fatty acid analysis in particular, for caring about my research, and for having a detailed answer for every one of my questions.

To Kumar, for introducing to me and teaching me the secrets of successful molecular biology research. Thank you for your patience and for your kindness.

To my past and present friends in the lab. Thank you, Holly, my office mate, for putting up with having me (and sometimes my children) around, and for all of your very helpful advice. Thank you, Andrea and Maggie, for being great friends and wonderful “aunts” to my kids. Thank you, Ying and Cynthia, for consistently lifting

my spirits.

To my friends, Elad and Asya, for your friendship, which has been so important to me, for sharing your knowledge and experience, and most of all for being a family to us.

To my sisters-in-law, Paula and Wendy, for your encouragement, your help, and your love.

To my parents, Ahron and Ofra, and to my sister, Amit, who despite the geographical distance felt so close. Thank you for teaching me always to go forward and never stop in one place, even if it has meant being so far away from you. I love you, and I miss you very much.

To Tomer and Noa, my very own vernix producers, I do not have the words to express how much I love you and how happy and proud you make me. Your understanding, your patience, and your support for me during the last five years were remarkable.

Lastly, to my beloved husband, Billy, who since the very first day we met, never failed believing in me, and ever since has played a tremendous role in helping to make me who I am. None of this could have happened without you. Thank you for always being there for me, for your wise advice, for your understanding, and for caring about my work and my interests. I love you so much.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BCFA, branched chain fatty acids

CLA, conjugated linoleic acid

DF, Dam-fed

DHA, docosahexaenoic acid

EPA, eicosapentaenoic acid

FA, fatty acids

FAME, fatty acid methyl esters

GC, gas chromatography

GI, gastrointestinal

IFN-γ, interferon-γ

MS, mass spectrometry

Muc, mucin

MUFA, monounsaturated fatty acids

NEC, necrotizing enterocolitis

PL, phospholipids

SE, sterol esters

SFA, saturated fatty acids

TAG, triacylglycerol

TFF3, Trefoil factor- 3

TGF-β, transforming growth factor - β

TNF-α, tumor necrosis factor-α

US, United States

WE, wax esters

CHAPTER 1

BRANCHED CHAIN FATTY ACIDS ARE CONSTITUENTS OF THE NORMAL HEALTHY NEWBORN GASTROINTESTINAL TRACT*[1]

Abstract

Vernix suspended in amniotic fluid is normally swallowed by the late term fetus. We hypothesized that branched chain fatty acids (BCFA), long known to be major vernix components, would be found in meconium and that the profiles would differ systematically. The objective of the study was to investigate the presence of BCFA in meconium and to characterize their relative profiles in both vernix and meconium. Vernix and meconium were collected from term newborns and analyzed. BCFA-containing lipids constituted about 12% of vernix dry weight, and were predominantly saturated, and had 11 to 26 carbons per BCFA. In contrast, meconium BCFA had 16 to 26 carbons, and were about 1% of dry weight. Meconium BCFA were mostly in the *iso*- configuration, whereas vernix BCFA contained dimethyl and middle chain branching, and five *anteiso* -BCFA. The mass of BCFA entering the fetal gut as swallowed vernix particles is estimated to be 180 mg in the last month of gestation while the total mass of BCFA found in meconium is estimated to be 16 mg, thus most BCFA disappear from the fetal gut. The BCFA profiles of vernix and meconium show that BCFA are major components of normal healthy term newborn gastrointestinal tract. BCFA are candidates for agents that play a role in gut colonization and should be considered a nutritional component for the fetus/newborn.

* Ran-Ressler RR, Devapatla S, Lawrence P, Brenna JT 2008 Branched chain fatty are constituents of the normal healthy newborn gastrointestinal tract. Pediatr Res 64:605-609.

Introduction

Branched chain fatty acids (BCFA) are mostly saturated fatty acids (SFA) with one or more methyl branches on the carbon chain. The structure of the predominant monomethyl BCFA of the *iso*- and *anteiso*-configuration is shown in **Figure 1.1**.

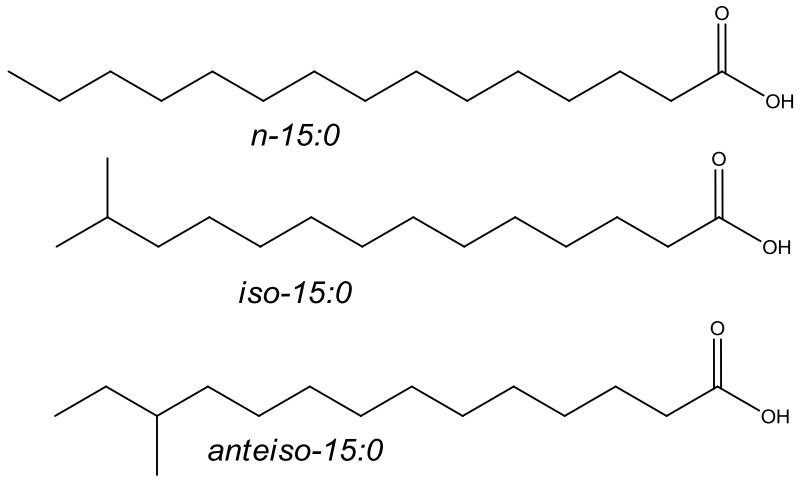

Figure 1.1 Structures of representative branched chain fatty acids (BCFA). *n- (normal)* hydrocarbon chains are straight with no branching. *iso*-BCFA have a bifurcated methyl branch. The systematic name for *iso*-15:0 is 13-methyl tetradecanoic acid. *anteiso*-BCFA have a methyl branch on the antepenultimate carbon. *anteiso*-15:0 is 12-methyl tetradecanoic acid.

BCFA are synthesized mainly by the skin and have long been known to be a major component of vernix caseosa (10-20% dry weight) (1). Among terrestrial animals, vernix is unique to humans, and is not found in other land mammals, including other primates (2). Vernix is made of sebum and fetal corneocytes (1, 3) and is produced by

fetal skin starting at 24 weeks gestational age and continuing until term birth (4). During the third trimester vernix sloughs off as particulates that become suspended in amniotic fluid (3, 5), possibly aided by lung surfactant phospholipids that also enter the amniotic fluid. The fetus normally swallows amniotic fluid in amounts approaching 500 ml at the end of gestation (6, 7) and with it vernix. Thus, the late term fetal gut is normally exposed to vernix and its BCFA, increasingly so as parturition approaches.

Vernix dry matter is composed of approximately equal amounts of protein and lipids (2, 8). Lipid fractions in vernix have been comprehensively characterized (9-11) and shown to be 25-30% sterol esters (SE), 18-36% triglycerides (TAG), 12-16% wax esters (WE), 9% squalene, 5% ceramides, and low levels of non-esterified fatty acid (NEFA) fraction was also detected by some (10, 12) but not by others (13). BCFA are found in all acyl-carrying lipid classes, WE (16-53%) and SE (27-62%) (9-11, 13), as well as in the TAG (18-21%) and NEFA (21%) fractions (10).

Apart from skin (1, 9, 14), BCFA are at very low levels in internal tissue (14), but are also found in human milk (15-17) at concentrations as high as 1.5%w/w of total fatty acids (FA). This level is comparable to and in some cases greater than that of docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) in the same milk. For instance, a 1981 publication reported the concentration of *anteiso*-17:0 in Australian women's colostrum to be 0.45%w/w of total FA, exceeding the concentrations of DHA (0.32%w/w) and ARA (0.4%w/w) (17).

Meconium, the newborn's first fecal pass, first appears in the fetal GI tract at around 12 weeks of gestational age, and is normally passed after birth (18-20). It consists of

amniotic fluid residue, skin and gastrointestinal (GI) epithelial cells, GI secretions and enzymes, lipids, sugars, proteins, cholesterol, sterols, bile acid and salts (18, 19, 21, 22). Meconium contains 12% dry weight lipid (21), and there is only one unconfirmed study reporting BCFA in meconium (23). There are no studies linking BCFA composition of vernix and meconium in the same infants.

We hypothesized that vernix BCFA of term newborns would survive the alimentary canal and be found in meconium. Our test of this hypothesis led us to characterize the relative BCFA profiles of vernix and meconium to establish the degree to which the profile is altered by the sterile fetal gut in utero.

Methods

This study was approved by the Cornell University and the Cayuga Medical Center Institutional Review Boards (IRB) on the Use of Human Subjects. The IRB approved an exemption from the requirement to obtain individual informed consent because the human materials sampled, vernix and meconium, are deemed to be medical waste and no individually identifiable information was obtained from participants.

Sample collection. Eighteen samples of vernix and meconium were collected from 18 normal term newborns at Cayuga Medical Center in Ithaca, NY. Vernix was removed from the shoulder regions in the birthing room, placed in clean tubes and stored at -80°C until analysis. Meconium was collected from diapers and similarly transferred into clean tubes and stored at -80°C until analysis.

FA analysis. Total lipids were extracted from the vernix and the meconium samples according to a modified Bligh and Dyer method (24). Fatty acids are overwhelmingly found in mammalian pools, such as vernix and meconium, as acyl moieties which are constituents of higher molecular weight lipid molecules such as TAG, SE, and WE. For detailed molecular analysis, fatty acyl groups are hydrolyzed and fatty acid methyl esters (FAME) synthesized for analysis. FAME were prepared using 14% BF3 in methanol (Sigma Chemical, St. Louis, MO.). Butylated hydroxytoluene (BHT) was added to methanol as an antioxidant. Heptadecanoic acid (Sigma Chemical, St Louis, MO) in chloroform was used as an internal standard. This routine step obscures heptadecanoic acid which is normally rare in mammalian tissue but is present in vernix and meconium. Because of the extraordinary diversity of FA in these samples, any internal standard interferes with analysis of one or more FA in some of the samples. A correction was applied to estimate the extent of interference, and the signals were carefully calibrated against external standards.

FAME analyses were performed using a Hewlett Packard 5890 series II gas chromatograph (GC). A BPX-70 column (60m×0.32mm×0.25µm, SGE, Austin, Tx) was used for the analysis with H_2 as the carrier gas. FAME identities were determined by a chemical ionization (CI) and electron impact (EI) mass spectrometry (MS), using a Varian Star 3400 GC coupled to a Varian Saturn 2000 ion trap MS. BCFA FAME identities were based on GC retention time of each substance and its electron impact mass spectra. FAME mass spectral assignments were confirmed by conversion of the FAME to picolinyl ester derivatives according to the method described by Christie <www.lipidlibrary.co.uk> and Yang (25), followed by GC/MS analysis and comparison to literature spectra (26-31).

An equal weight FAME mixture (68A; Nuchek Prep, Elysian, MN) was used to calculate response factors. The following were also used as standards: n-11:0 up to n-24:0 (Nuchek Prep, Elysian, MN); *iso*- 13:0, *anteiso*- 13:0, *iso*- 15:0, *anteiso*- 15:0; *iso*- 17:0, *anteiso*- 17:0 (Larodan Fine Chemicals AB, Malmo, Sweden) and 10 methyl hexadecanoic acid (Matreya LLC, Pleasant Gap, PA). FA levels were expressed as weight % of total fatty acids for all 11 to 32 carbons FA.

Statistics. Data are expressed as mean±SD for study population characteristics, and as mean±SEM for FA analysis. Statistical analyses were made using JMP 6 (SAS Institute, Cary, NC). Differences in mean of each FA were calculated using one sample t-test for non-zero differences, with $p<0.05$ declared significant.

Results

Subjects. Characteristics of the study population are presented in **Table 1.1**. No complications were present for any of the newborns other than as noted. All but two newborns were by vaginal delivery. Six mothers received antibiotic treatment during pregnancy; five of them gave birth to female infants.

Table 1.1. Characteristics of study population

	Mean ± SD	Range
Mother's age (years)	29±5.8	18-42
Gestational age (weeks)	40±1	38-41
Birthweight (kg)	3.3±0.5	2.3-4.4
Gender	10 females, 8 males	
Delivery	16 vaginal, 2 CS*	
Antibiotics	5 females, 1 male	

* *Cesarean section*

Overall FA distribution. A profile of FA classes is shown in **Table 1.2**. Comparisons of all classes were significant at the $p<0.05$ level. BCFA constituted almost a third (29.1±1.5%w/w) of all FA in vernix and were significantly higher compared to the mean levels in meconium (17.5±1.3%w/w; $p<0.05$). This drop in BCFA was accompanied by a reciprocal increase in normal (n-) saturated FA (n-SFA) specifically, 34±1.9%w/w in vernix and 51.3±3.0%w/w in meconium ($p<0.05$). Differences in *n*-monounsaturated fatty acids *(*MUFA) and polyunsaturated fatty acid (PUFA) were modest by comparison.

Table 1.2. Profile of fatty acid classes (%w/w) in vernix and meconium (mean±SEM)

FA	Vernix	Meconium
BCFA	29.1±1.5*	17.5±1.3
n-SFA	34±1.9*	51.3±3.0
n-MUFA	31.0±1.7*	22.4±2.1
PUFA	3.9±0.4*	7.1±1.1
*p<0.05		

Overall, BCFA hydrolyzed from their native lipid classes constituted 5.8% of dry weight of vernix, corresponding to approximately 12% of dry weight of vernix within the native BCFA-containing lipids. Meconium had 0.55% dry weight of hydrolyzed BCFA and an estimated 1% of BCFA-containing lipids.

BCFA distribution in vernix and meconium. **Figure 1.2** is a graphical summary of the BCFA profiles for vernix and meconium for those BCFA detected in samples from at least 3 newborns, presented left to right in order of carbon number. In total, 30 BCFA were identified in vernix while nine were also detected in meconium. Vernix BCFA ranged from 11 to 26 carbon atoms and were primarily saturated apart from two *iso*- monounsaturates. *Iso*- BCFA, *anteiso*- BCFA, middle chain monomethyl

BCFA and dimethyl BCFA were all detected among vernix BCFA. In contrast, meconium BCFA had a much more restricted range of carbon numbers, from 16 to 26 carbons. Of the nine meconium BCFA, eight were *iso*- BCFA, of which two were MUFA, and one was *anteiso*.

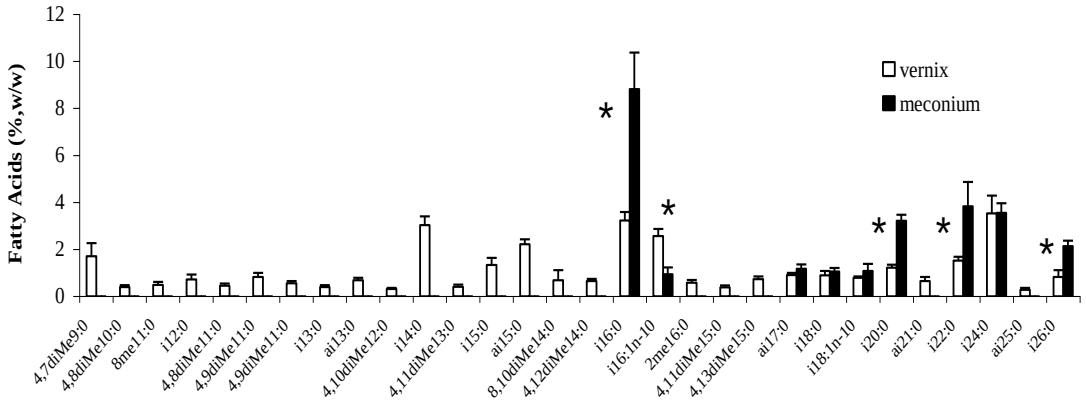

Figure 1.2 Branched chain fatty acid (BCFA) methyl ester profiles for vernix and meconium (means ± SEM, n≤18 newborn) listed left to right in order of molecular weight. Means are for those FA appearing in samples from at least three newborns. *iso*-BCFA have a dimethyl terminal structure: *iso*-16:0 is synonymous with 14-methyl-15:0 (14-methyl-pentadecanoic acid). *anteiso*-BCFA have a methyl branch at the n-2 position: anteiso-17:0 is synonymous with 14-methyl-16:0 (14-methyl-hexadecanoic acid). *i=iso*; *ai=anteiso*; Me=methyl; diMe = two methyl branches.

Key: □ vernix; ■ meconium. *p<0.05

The profile of the *iso*- BCFA in vernix and meconium is shown in **Figure 1.3.** The vernix *iso*- BCFA profile had odd and even carbon numbered FA from *iso*-12:0 to *iso*-16:0, and only even carbon numbers at greater chain lengths. In contrast, meconium *iso* BCFA was dominated by the shortest chain BCFA in its profile, *iso*-16:0, which was more than twice the relative concentration of any other BCFA. Five of the eight *iso*-BCFA appearing in both vernix and meconium were a significantly different proportion of BCFA in the respective profiles; the preponderance of longer chains in meconium lead to significant differences in three of the four *iso*-BCFA of chain numbers from 20 to 26 carbons.

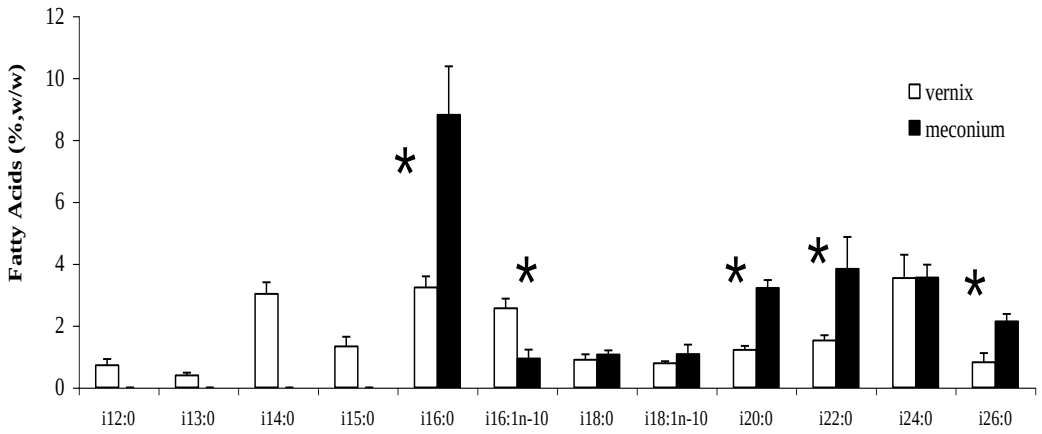

Figure 1.3. *iso*- Branched chain fatty acid (BCFA) methyl esters in vernix and meconium (means ± SEM, n≤18 newborn) listed left to right in order of molecular weight. *i=iso*. Key: □ vernix; ■ meconium. *p<0.05

The profile of *anteiso*- BCFA is shown in **Figure 1.4**. All five *anteiso*- BCFA detected in vernix are odd carbon numbered. They range from 13 to 25 carbons, and only one, *anteiso*-17:0, is found in meconium.

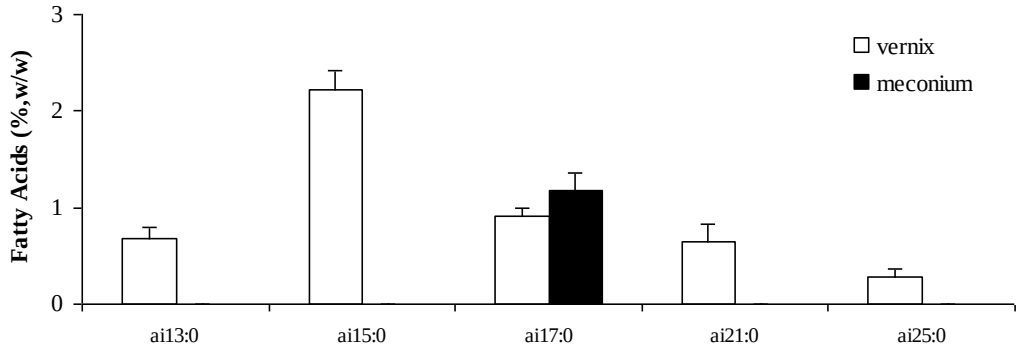

Figure 1.4. *anteiso*- Branched chain fatty acid (BCFA) methyl esters in vernix and meconium (means ± SEM, n≤18 newborn) listed left to right in order of molecular weight. *ai=anteiso*. Key: □ vernix; ■ meconium. *p<0.05

Figure 1.5 is a graphical summary of the straight chain, *n*-FA, profiles for vernix and meconium. Vernix normal FA had 11 to 26 carbon atoms, and meconium FA had 14 to 26 carbons, and both contained small amounts of odd chain number FA. Again, meconium BCFA tended to be of greater molecular weight.

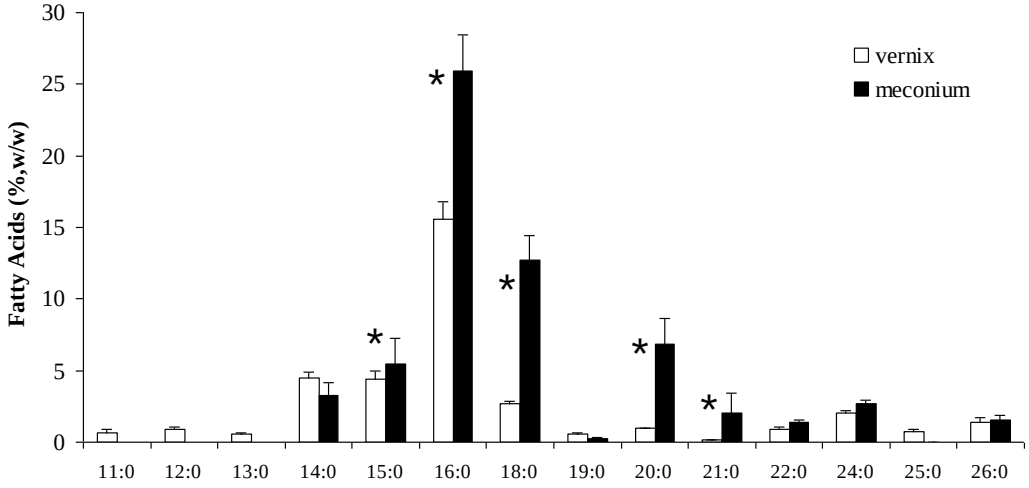

Figure 1.5. Normal (straight chain) fatty acid methyl esters in vernix and meconium (means ± SEM, n≤18 newborn) listed left to right in order of molecular weight.

Key: □ vernix; ■ meconium. *p<0.05.

Discussion

The presence of BCFA in both vernix and meconium of healthy term infants indicates that BCFA are a major component of gut contents of normal term newborns, and their presence in meconium implies that they are present throughout the length of the gut. As such, BCFA are a component of the GI tract milieu present when the first few environmental microorganisms appear in the initial stage of gut colonization during and immediately after parturition or Cesarean section. In meconium, the systematic

shift in BCFA profiles to high molecular weights, as well as the absence of most BCFA other than *iso*-BCFA, indicates that the fetal alimentary canal readily absorbs and metabolizes most BCFA.

There are many reports of BCFA in breastmilk, with the earliest and most extensive, showing 54 BCFA with a cumulative concentration of 1.5%w/w (16). A 1981 paper measured the concentration of BCFA in mature Australian breast milk to be a total of 0.84%w/w and one BCFA, *anteiso*-17:0 in colostrum at 0.45%w/w of fatty acids, exceeding the concentrations of DHA (0.32%w/w) and ARA (0.40%w/w) in the same mother's mature breastmilk (17). Chen reported four BCFA with a cumulative concentration of 0.58%, w/w in Canadian breastmilk (32). A study of California women yielded an average of 0.60% BCFA for the four BCFA that were reported (15:0, 16:0, 17:0, 18:0, branch location not reported) (33). Corso found six BCFA in milks of 40 women in southern Italy (34), with *anteiso*-17:0 ranging from 0.12 to 0.93% of total fatty acids. This variability is not unlike that for DHA; breastmilk DHA ranges between 0.06%w/w and 1.40%w/w (35). Many, but not all, breastmilk fatty acid concentrations are closely linked to the dietary intake of the fatty acid or its precursor, including DHA to which the wide reported range is ascribed (35). It is most likely that the absence of BCFA identification in most breast milk FA papers is due to their low concentration, their appearance in a GC trace amidst the major saturates and monounsaturates in the chromatogram, and the historical absence of a compelling metabolic role for them.

The exposure of the gut to BCFA *in utero*, and possibly from breastmilk, is greater than any other period of life because BCFA are at trace levels in normal foods. The unique niche represented by BCFA and other components in the human gut may be

important for establishing commensal bacteria during colonization. BCFA are prominent membrane components of many bacterial species (36, 37). For instance, BCFA constitute 95% of the FA in several bacilli and lactobacilli, including *Sporolactobacillus inulinus*, which has very recently been shown to be a probiotic candidate (37). The FA of nine *Bifidobacterium* strains include BCFA such as *iso*-14:0, *anteiso*-15:0, *iso*-16:0 and *iso*-18:0 at various levels (0.6-24.6%w/w). *iso*-14:0 is the second most abundant FA in *Bifidobacterium breve* with levels as high as 24.6%w/w (38). It is reasonable to hypothesize that the presence of BCFA in the neonatal gut would alter the mix of dominant species, favoring those organisms that use BCFA in their membranes, and we postulate that BCFA are a unique feature of the human fetal gut favoring the growth of commensal bacteria during colonization.

This hypothesis has implications for colonization of the GI tract of very premature infants, and may be a factor in the development of necrotizing enterocolitis (NEC), the etiology and pathogenesis of which is not well understood (39, 40). NEC is one of the major causes of morbidity in premature infants (39) though it is certainly related to pathogen overgrowth (41). Leading hypotheses with empirical support are that NEC is related to prematurity, enteral feeding, and bacterial colonization (40). Importantly, it has not been observed prenatally. NEC risk is higher among lower gestational age infants and is rare in term infants (42). Breast milk consumption is associated with a lower incidence of NEC (40, 43). Although no specific pathogenic bacteria has been associated with NEC (43), supplementation of premature animals and infants with probiotic strains appear to reduce its incidence (39, 44). With these considerations, we hypothesize that BCFA have a role in enhancing proper GI colonization: vernix begins to appear around week 24 of gestation and accumulates as particulates in amniotic fluid toward term (3) thus, the GI tract of very premature infants is not exposed to

vernix BCFA prenatally. Postnatally they would be exposed to BCFA if breastfed, but formula-fed preterms would not be exposed to BCFA since they are not a component of preterm formulas. Finally, we note that the incidence of NEC drops as gestational age approaches normal term, therefore later term premature infants would be exposed to some BCFA and may benefit if the hypothesis is correct.

We can estimate the mass of BCFA entering and exiting the alimentary canal. At term, amniotic fluid lipids are about 154 mg/L (45), of which about 52 mg/L are phospholipids that are likely to originate as BCFA-free lung surfactant (3, 10). Thus, the amniotic fluid vernix lipids concentration is about 102 mg/L. Of this, our measurements indicate that 57% are FA, to yield 58 mg/L. Our data (Table 2) further indicate that 29% are BCFA, to yield 17 mg/L BCFA. The fetus is estimated to swallow 200 to 500 ml/day of amniotic fluid near term (46, 47), and taking the midpoint of this range, 350 ml/day, 6 mg BCFA per day enter the fetal GI tract amounting to 30×6 = 180 mg BCFA in the last month of gestation. Meconium is the output of the GI tract integrated from about 12 weeks gestation. Total meconium for 27 term infants was reported (48) to be 8.9 g wet weight, averaging 32% dry weight, or 2.8 g. Our data indicate that about 0.55% is BCFA, or about 16 mg average total BCFA in meconium. This value is an order of magnitude lower than our estimate of the BCFA swallowed in the last month of gestation, and suggests that most of the BCFA disappear during transit. The distribution and structural characteristics of BCFA that do appear in meconium reflect processing of vernix by the enterocytes. Our data show that C12-15 BCFA, as well as nearly all BCFA apart from *iso*-BCFA, are absent from meconium and thus must have been metabolized. The nature of this metabolism remains to be determined, in part because BCFA and their interaction with human enterocytes have not been studied.

Chain elongation is one likely metabolic transformation that would explain the absence of C12-15 BCFA, and preponderance of longer chain BCFA, in meconium. Suggestive evidence in support of this hypothesis is found in the data of Figure 1. The significantly greater level of meconium *iso*-16:0 compared to vernix *iso*-16:0, is roughly the sum of vernix *iso*-14:0 and *iso*-16:0, consistent with the hypothesis that elongation of vernix *iso*-14:0 adds to the existing *iso*-16:0. Similar observations apply to meconium *iso*-20:0 and vernix *iso*-18:0 and *iso*-20:0.

Medium chain fatty acids (C8-C14) are commonly fed to premature infants because they are efficiently absorbed through the gastric mucosa, directly transported to the liver via the portal vein, and oxidized by the immature GI tract. Although the BCFA with 15 or fewer carbons are absent from meconium, Figure 1.4 shows that the FA *n*-14:0 and *n*-15:0 are partially excreted. This observation implies that there is selective uptake and retention of BCFA by the fetal GI tract that may not operate as efficiently for the *n*-FA.

Our measurements of BCFA are in line with previous data. BCFA constituted almost one third of all FA in vernix (11, 49), and the levels of vernix SFA, MUFA and PUFA were within the range encompassed by previous reports (10, 49, 50). We found only odd numbered carbon *anteiso* BCFA, consistent with some previous reports (49-51), but not with others (10, 11). BCFA averaged 17%w/w of all FA in meconium in our samples. The single previous study showing BCFA in meconium reported only on the free fatty acid fraction and used GC with retention time matching for identification. *iso* FA with 22 and 24 carbons were identified at 4%w/w and 6%w/w respectively,

and nine other *iso*-BCFA were tentatively assigned (C14-21, 25) with no percent fraction provided.

Though five *anteiso*- BCFA were detected in vernix, *anteiso*-17:0 was the sole *anteiso*-BCFA detected in meconium, and there is no obvious explanation as to why this was the case. Weanling rats fed 100mg/week *anteiso*-17:0 in an otherwise fat free diet excreted 8-10% in the feces and stored a similar amount in adipose tissue (52), and apparently also converted a small amount to *anteiso*-15:0. The remaining 80% was metabolized to substances other than *anteiso*- FA. The levels of *anteiso*-17:0 have been reported to be the highest among all BCFA in at least one study of breastmilk (17), and it is notable that *anteiso*-17:0 is a major lipids constituent of many bacterial membrane (36).

The combined levels of the middle chain monomethyl and dimethyl BCFA in our vernix samples were similar to the levels reported in a single vernix sample by Nicolaides & Apon (49). In our sample of 18 newborns, the average proportions of dimethyl monomethyl BCFA dominated over middle chain monomethyl BCFA. The first methyl branch in the dimethyl BCFA was located predominantly on the fourth carbon of the chain, consistent with previous findings (49, 50). However, in our study, the second methyl branch in half of the dimethyl BCFA was located on an odd numbered carbon, and in almost all the dimethyl BCFA, this methyl branch was located on the *anteiso* carbon of the FA chain.

In summary, there are dramatic and systematic differences in BCFA composition between vernix and meconium, indicating that BCFA are actively metabolized in the

fetal GI tract. This observation implies that vernix should be considered a nutritional agent, and that BCFA are a normal and quantitatively substantial component of the normal term newborn GI tract. Further studies are warranted to understand the uptake and metabolism of BCFA by enterocytes, and the role of BCFA during bacterial colonization. The absence of vernix, and BCFA, in the GI tract of very premature, formula-fed infants may have a role in the etiology of NEC, among the most devastating conditions facing the preterm infant.

REFERENCES

1. Nicolaides N, Ray T 1965 Skin Lipids. 3. Fatty Chains in Skin Lipids. the Use of Vernix Caseosa to Differentiate between Endogenous and Exogenous Components in Human Skin Surface Lipid. J Am Oil Chem Soc 42:702-707
2. Pickens WL, Warner RR, Boissy YL, Boissy RE, Hoath SB 2000 Characterization of vernix caseosa: water content, morphology, and elemental analysis. J Invest Dermatol 115:875-881
3. Narendran V, Wickett RR, Pickens WL, Hoath SB 2000 Interaction between pulmonary surfactant and vernix: a potential mechanism for induction of amniotic fluid turbidity. Pediatr Res 48:120-124
4. Moore C, Dempsey D, Deitermann D, Lewis D, Leikin J 1996 Fetal cocaine exposure: analysis of vernix caseosa. J Anal Toxicol 20:509-511
5. Yoshio H, Tollin M, Gudmundsson GH, Lagercrantz H, Jornvall H, Marchini G, Agerberth B 2003 Antimicrobial polypeptides of human vernix caseosa and amniotic fluid: implications for newborn innate defense. Pediatr Res 53:211-216
6. Miettinen TA, Luukkainen T 1968 Gas-liquid chromatographic and mass spectrometric studies on sterols in vernix caseosa, amniotic fluid and meconium. Acta Chem Scand 22:2603-2612
7. Sherman DJ, Ross MG, Day L, Ervin MG 1990 Fetal swallowing: correlation of electromyography and esophageal fluid flow. Am J Physiol 258:R1386-1394

8. Hoeger PH, Schreiner V, Klaassen IA, Enzmann CC, Friedrichs K, Bleck O 2002 Epidermal barrier lipids in human vernix caseosa: corresponding ceramide pattern in vernix and fetal skin. Br J Dermatol 146:194-201
9. Nicolaides N, Fu HC, Ansari MN, Rice GR 1972 The fatty acids of wax esters and sterol esters from vernix caseosa and from human skin surface lipid. Lipids 7:506-517
10. Rissmann R, Groenink HW, Weerheim AM, Hoath SB, Ponec M, Bouwstra JA 2006 New insights into ultrastructure, lipid composition and organization of vernix caseosa. J Invest Dermatol 126:1823-1833
11. Kaerkkaeinen J, Nikkari T, Ruponen S, Haahti E 1965 Lipids of Vernix Caseosa. J Invest Dermatol 44:333-338
12. Tollin M, Bergsson G, Kai-Larsen Y, Lengqvist J, Sjovall J, Griffiths W, Skuladottir GV, Haraldsson A, Jornvall H, Gudmundsson GH, Agerberth B 2005 Vernix caseosa as a multi-component defence system based on polypeptides, lipids and their interactions. Cell Mol Life Sci 62:2390-2399
13. Nazzaro-Porro M, Passi S, Boniforti L, Belsito F 1979 Effects of aging on fatty acids in skin surface lipids. J Invest Dermatol 73:112-117
14. Nicolaides N 1974 Skin lipids: their biochemical uniqueness. Science 186:19-26
15. Jensen RG 1995 Handbook of Milk Composition. Academic Press Inc., San Diego
16. Egge H, Murawski U, Ryhage R, Gyorgy P, Chatranon W, Zilliken F 1972 Minor constituents of human milk. IV. Analysis of the branched chain fatty acids. Chem Phys Lipids 8:42-55
17. Gibson RA, Kneebone GM 1981 Fatty acid composition of human colostrum and mature breast milk. Am J Clin Nutr 34:252-257.

18. Ahanya SN, Lakshmanan J, Morgan BL, Ross MG 2005 Meconium passage in utero: mechanisms, consequences, and management. Obstet Gynecol Surv 60:45-56; quiz 73-44
19. Gareri J, Klein J, Koren G 2006 Drugs of abuse testing in meconium. Clin Chim Acta 366:101-111
20. Ostrea EM, Jr., Hernandez JD, Bielawski DM, Kan JM, Leonardo GM, Abela MB, Church MW, Hannigan JH, Janisse JJ, Ager JW, Sokol RJ 2006 Fatty acid ethyl esters in meconium: are they biomarkers of fetal alcohol exposure and effect? Alcohol Clin Exp Res 30:1152-1159
21. Buchanan DJ, Rapoport S 1952 Chemical comparison of normal meconium and meconium from a patient with meconium ileus. Pediatrics 9:304-310
22. Righetti C, Peroni DG, Pietrobelli A, Zancanaro C 2003 Proton nuclear magnetic resonance analysis of meconium composition in newborns. J Pediatr Gastroenterol Nutr 36:498-501
23. Terasaka D, Clark DA, Singh BN, Rokahr J 1986 Free fatty acids of human meconium. Biol Neonate 50:16-20
24. Bligh EG, Dyer WJ 1959 A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911-917
25. Serhan CN, Gotlinger K, Hong S, Lu Y, Siegelman J, Baer T, Yang R, Colgan SP, Petasis NA 2006 Anti-inflammatory actions of neuroprotectin D1/protectin D1 and its natural stereoisomers: assignments of dihydroxy-containing docosatrienes. J Immunol 176:1848-1859
26. Carballeira NM, Miranda C, Lozano CM, Nechev JT, Ivanova A, Ilieva M, Tzvetkova I, Stefanov K 2001 Characterization of novel methyl-branched chain fatty acids from a halophilic Bacillus species. J Nat Prod 64:256-259

27. Suutari M L, S. 1993 Signature GLC-MS ions in identification of delta 5 and delta 9 unsaturated iso and anteiso branched chain fatty acids. J Microbiol Methods 17:39-48

28. Karlsson Hasse OG 1969 Studies on feather waxes of birds. VIII. Arkiv For Kemi 31:143-158

29. Yu QT, Liu BN, Zhang JY, Huang ZH 1988 Location of methyl branchings in fatty acids: fatty acids in uropygial secretion of Shanghai duck by GC-MS of 4,4-dimethyloxazoline derivatives. Lipids 23:804-810

30. Apon JM, Nicolaides N 1975 The determination of the position isomers of the methyl branches fatty acids methyl esters by capillary GC/MS. J Chromatogr Sci 13:467-473

31. Harvey DJ 1989 Identification of long-chain fatty acids and alcohols from human cerumen by the use of picolinyl and nicotinate esters. Biomed Environ Mass Spectrom 18:719-723

32. Chen ZY, Pelletier G, Hollywood R, Ratnayake WM 1995 Trans fatty acid isomers in Canadian human milk. Lipids 30:15-21

33. Aitchison JM, Dunkley WL, Canolty NL, Smith LM 1977 Influence of diet on trans fatty acids in human milk. Am J Clin Nutr 30:2006-2015

34. Corso G, Colavita C, Esposito M, Roma R, Napoli C, Zamparelli M, Ansanelli V 1995 Gaschromatography-mass spectrometry analysis of fatty acids in human milk from forty puerperae living in southern Italy. Riv Eur Sci Med Farmacol 17:215-219

35. Brenna JT, Varamini B, Jensen RG, Diersen-Schade DA, Boettcher JA, Arterburn LM 2007 Docosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. Am J Clin Nutr 85:1457-1464

36. Kaneda T 1991 Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol Rev 55:288-302
37. Huang HY, Huang SY, Chen PY, King VA, Lin YP, Tsen JH 2007 Basic characteristics of Sporolactobacillus inulinus BCRC 14647 for potential probiotic properties. Curr Microbiol 54:396-404
38. Veerkamp JH 1971 Fatty acid composition of Bifidobacterium and Lactobacillus strains. J Bacteriol 108:861-867
39. Caplan MS, Miller-Catchpole R, Kaup S, Russell T, Lickerman M, Amer M, Xiao Y, Thomson R, Jr. 1999 Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Gastroenterology 117:577-583
40. Claud EC, Walker WA 2001 Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. Faseb J 15:1398-1403
41. Hallstrom M, Eerola E, Vuento R, Janas M, Tammela O 2004 Effects of mode of delivery and necrotising enterocolitis on the intestinal microflora in preterm infants. Eur J Clin Microbiol Infect Dis 23:463-470
42. Beeby PJ, Jeffery H 1992 Risk factors for necrotising enterocolitis: the influence of gestational age. Arch Dis Child 67:432-435
43. Lucas A, Cole TJ 1990 Breast milk and neonatal necrotising enterocolitis. Lancet 336:1519-1523
44. Hoyos AB 1999 Reduced incidence of necrotizing enterocolitis associated with enteral administration of Lactobacillus acidophilus and Bifidobacterium infantis to neonates in an intensive care unit. Int J Infect Dis 3:197-202

45. Biezenski JJ, Pomerance W, Goodman J 1968 Studies on the origin of amniotic fluid lipids. I. Normal composition. Am J Obstet Gynecol 102:853-861

46. Pritchard JA 1965 Deglutition by Normal and Anencephalic Fetuses. Obstet Gynecol 25:289-297

47. Pritchard JA 1966 Fetal swallowing and amniotic fluid volume. Obstet Gynecol 28:606-610

48. Friel JK, Matthew JD, Andrews WL, Skinner CT 1989 Trace elements in meconium from preterm and full-term infants. Biol Neonate 55:214-217

49. Nicolaides N, Apon JM 1976 Further studies of the saturated methyl branched fatty acids of vernix caseosa lipid. Lipids 11:781-790

50. Nicolaides N 1971 The structures of the branched fatty acids in the wax esters of vernix caseosa. Lipids 6:901-905

51. Haahti E, Nikkari T, Salmi AM, Laaksonen AL 1961 Fatty acids of vernix caseosa. Scand J Clin Lab Invest 13:70-73

52. Livingston M, Bell ME, Shorland FB, Gerson T, Hansen RP 1957 The metabolism in the rat of naturally occurring (+)-14-methylhexadecanoic acid. Biochem J 65:438-440

CHAPTER 2

BRANCHED CHAIN FATTY ACIDS REDUCE THE INCIDENCE OF NECROTIZING ENTEROCOLITIS IN A NEONATAL RAT MODEL*[1]

Abstract

Branched chain fatty acids (BCFA) are major components of vernix caseosa. Vernix suspended in late gestation amniotic fluid is swallowed by the fetus, and BCFA are found as normal components of the newborn gastrointestinal (GI) tract. We tested the hypothesis that premature infant lack of GI BCFA exposure could be linked to the risk for necrotizing enterocolitis (NEC) in an established animal model. Three groups of premature rats were dam-fed (DF), or hand fed either a rat milk substitute (NEC) or a rat milk substitute with 20% w/w BCFA (BCFA). All groups were exposed to asphyxia and cold stress to develop NEC. Intestinal injury, cytokines and mucins gene expression, and ileal BCFA uptake were evaluated. NEC incidence was reduced by more than 50% in the BCFA group compared to the NEC group as assessed in ileal tissue. BCFA were selectively incorporated into ileal phospholipids. IL-10 mRNA was increased 3-fold in the BCFA group compared to the NEC group, and no other inflammatory or mucosal mRNA markers were altered. These data indicate that BCFA reduce NEC incidence, and are consistent with the hypothesis that the effect is at least in part mediated by enhanced IL-10 by BCFA.

*Ran-Ressler RR, Khailova L, Arganbright KM, Adkins-Rieck CK, Jouni Z, Brenna JT, Dvortak, B Branched chain fatty acids reduce the incidence of necrotizing enterocolitis in a neonatal rat model. Submitted to the Pediatric Research, August 2010.

Introduction

Branched chain fatty acids (BCFA) are primarily saturated fatty acids (SFA) with one or more methyl branches on the carbon chain. In mono-methyl BCFA, most methyl branching is at the penultimate *(iso)* or antepenultimate carbon *(anteiso*) as shown in **Figure 1.1** In humans, BCFA are synthesized mainly by the skin, and they are also found in colostrum and human milk (1, 2). In colostrum, BCFA have been reported up to 1.5%w/w, of fatty acids (FA) with *anteiso*-17:0 at 0.45%w/w, which is greater than the mean concentrations of docosahexaenoic acid (0.32%w/w) and arachidonic acid (0.4%w/w) (1). An early study reported more than 50 different BCFA in human milk, totaling 1.5%w/w (2), and a more recent study reported a single BCFA, *anetiso*-17:0, ranging from 0.12-0.93% of total FA (3). Thus, BCFA are a natural component of breastfed infant intake at levels similar to those fatty acids known to be highly bioactive.

BCFA synthesized by human skin (4, 5) comprise 25-30%w/w of vernix FA (6). Vernix production by the fetal skin begins at about the mid-way time through normal gestation (7) and continues until term birth (8). From early in the third trimester, vernix becomes suspended in amniotic fluid (8), and is swallowed by the fetus in increasingly large amounts as term birth approaches (8, 9). We recently showed that BCFA are present in meconium of healthy term infants (6), implying that they are present throughout the length of the gut. Moreover, BCFA with fewer than 16 carbons were detected in vernix but not in meconium, while BCFA with at least 16 carbons were detected in both. This selective shift in BCFA distribution indicates that the fetal alimentary canal metabolizes BCFA, suggesting that BCFA play a metabolic role in the developing gut.

Necrotizing enterocolitis (NEC) is a major cause of morbidity and mortality in premature infants (10). Its pathogenesis is unclear, and no effective prophylactic has emerged. Major risk factors for NEC development are prematurity, enteral feeding, abnormal bacterial colonization, and intestinal hypoxia-ischemia (11, 12). Human milk is associated with reduced NEC risk compared to formulas (10, 13, 14), most of which do not contain BCFA because they contain little to no cow's milk fat. The incidence of NEC drops as gestational age approaches normal term (15), consistent with the increase in BCFA gut exposure from ingested vernix. We speculate that development of NEC is related to the absence of BCFA, either from vernix, breast milk, or both.

In this study, we test the hypothesis that a pure BCFA mixture reduces the incidence of NEC in an established experimental model of NEC. A mixture of six *iso* and *anteiso* -BCFA substituted for 20%w/w of straight chain FA in normal rat pup feeds to simulate entry of BCFA via vernix. Ileal BCFA uptake in phospholipids (PL) and gene expression (mRNA) for several inflammation-related cytokines, for intestinal barrier-related mucins, and for trefoil factor 3 (TFF3) were measured to shed light on possible mechanisms mediating any observed effect.

Methods

Animal model and diets. The protocol was approved by the Animal Care and Use Committee of the University of Arizona (#A-324801-95081) and Cornell University. Seventy-three neonatal Sprague-Dawley rats (Charles River Laboratory, Pontage, MI) were collected by caesarian section one day before scheduled birth. Pups were assigned to one of three experimental groups: (a) dam-fed (DF, n=12), (b) hand-fed

with a rat's milk substitute formula (NEC, n=35), and (c) hand-fed with rat milk substitute prepared with 20%, w/w of BCFA mixture (BCFA, n=23).

The BCFA mixture was composed of six fatty acids obtained in purified, free fatty acid form (Larodan Fine Chemicals, Malmo, Sweden) at proportions similar to their mean proportions in vernix (6): *iso*-14:0 (25%), *anteiso*-15:0 (20%), *iso*-16:0 (25%), *anteiso*-17:0 (8%), *iso*-18:0 (10%) and *iso*-20:0 (12%). These were chosen because they represent the central range of BCFA found in vernix and are available commercially. The NEC diet was prepared as described previously (16), except that almond oil was used instead of sunflower oil. The BCFA diet was prepared as the NEC diet, except that (a) the fat emulsion was prepared as described by Baguma-Nibasheka (17), but with BCFA added such that the complete diet contained 20% w/w BCFA, and (b) soy oil was added instead of almond oil. The FA composition of the NEC and BCFA diets is shown in **Table 2.1**. Oleic acid (18:1n-9) and linoleic acid (18:2n-6) levels differ between the diets, due to the addition of BCFA. Each pup from the NEC and BCFA groups received a total of 850 µl of diet per day.

Table 2.1. Fatty acid composition (%w/w) of rat milk substitute (NEC), and rat milk substitute with BCFA (BCFA).

Fatty acids	NEC	BCFA
10:0	1.2	1.0
12:0	1.5	1.4
iso-14:0	0.1	4.9
14:0	4.8	4.8
14:1n-5	0.5	0.5
anteiso-15:0	0.2	3.9
15:0	0.5	0.5
iso-16:0	0.1	5.1
16:0	19.5	16.8
16:1	1.0	0.8
anteiso-17:0	0.2	1.6
17:0	0.3	0.3
iso-18:0	n.d.*	1.9
18:0	8.5	8.0
18:1n-9	30.7	21.5
18:2n-6	27.1	20.8
iso-20:0	n.d.*	2.5
18:3n-3	3.3	3.1
20:0	0.5	0.5
22:0	0.2	0.2

*n.d.- not detected

***NEC induction*.** NEC was induced in all groups according to existing methods (18, 19). Briefly, pups were exposed to a hypoxia challenge in 100% nitrogen gas twice daily for 1 min, followed by hypothermia-exposure at 4°C for 10 minutes. Pups were observed for signs of NEC, specifically abdominal distention, respiratory distress, and lethargy. Animals that developed signs of distress or imminent death before the end of the study were terminated and included in the study analysis. After 96 hours, all surviving pups were euthanized by decapitation. Animals expiring during the study were excluded from analysis.

For microscopic evaluation, 2 cm of distal ileum were removed after termination, fixed in 70% ethanol, embedded in paraffin and stained with hematoxylin and eosin in 4-6 μm sections. A histopathologist masked to the treatments assessed and graded the histological changes using intestinal injury scores developed previously (20): Grade 0, normal ileum; Grade 1, mild damage, slight submucosal and/or lamina propria separation; Grade 2, moderate to severe separation of the submucusa and/or lamina propria and/or edema in the submucosal and muscular layers, partial loss of villous sloughing; Grade 3, severe separation of the submucosa and/or lamina propria region, villous sloughing and initial villus necrosis; Grade 4, necrosis and loss of villi structure and/or transmural necrosis. Typical picture of these scores are shown in **Figure 2.1**. Half grade increments (+0.5, 1.5, 2.5 and 3.5) were used to more accurately assess levels of ileal damage. Animals with histological scores of grade 2 or more were considered to be NEC positive; this value is used because normal dam-fed animals which never develop NEC always are scored below this level. In addition, ileal tissue samples (20-35 mg) were collected from the NEC (n=4) and BCFA (n=3) groups for FA analysis of the PL fraction.

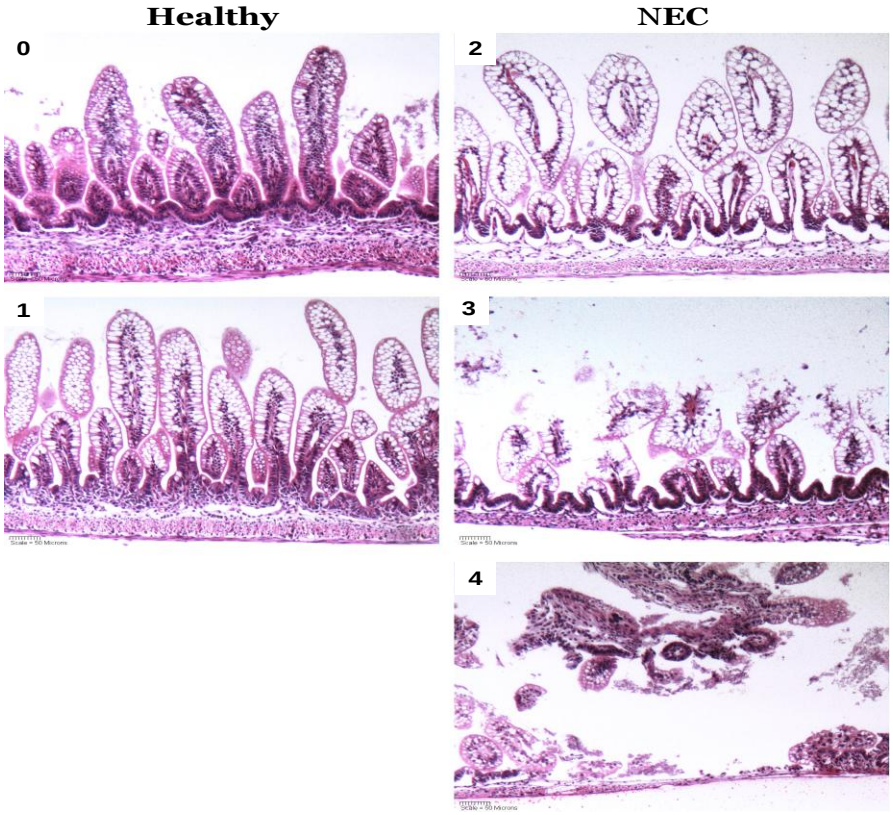

Figure 2.1 Histological scoring system of the terminal ileum of the neonatal rats. Histological changes were graded as follows: Score 0 – normal ileum; score 1 – mild damage with slight submucosal and/or lamina propria separation; score 2 – moderate to severe separation of submucosa and/or lamina propria, and/or edema in submucosal and muscular layers, partial villous sloughing; score 3 - severe separation of submucosa and/or lamina propria, region villous sloughing and initial villus necrosis; score 4 – necrosis and loss of villi structure and/or transmural necrosis.

RNA preparation & reverse transcriptase (RT) and real-time PCR. Total RNA was isolated from ileal tissue (snap frozen in liquid N_2) using the RNAeasy Plus Mini Kit (Qiagen, Santa Clarita, CA) as described in the manufacturer's protocol. Samples were incubated with RNase-free DNAse (20U/reaction) for 10 minutes at 37°C to eliminate DNA contamination. RNA concentration was quantified by ultraviolet (UV) spectrophotometry at 260 nm, and the purity was determined by the A260/A280 nm absorbance ratio (SPECTRAmax PLUS, Molecular Devices, Sunnyvale, CA). RNA integrity was verified by gel electrophoresis on a 1.2% agarose gel containing formaldehyde (2.2 mol/L) and ethidium bromide in 1X MOPS buffer [(40mM MOPS (pH 7.0), 10mM sodium acetate and 1 mM EDTA (pH 8.0)].

RT and real-time PCR assays were performed to quantify steady state mRNA levels of selected cytokines (IL-10, IL-18, IFN-γ, TNF-α, TGF-β), mucins (Muc 2, Muc 3, Muc 4) and trefoil factor 3 (TFF3). cDNA was synthesized from 0.5 μg of DNase-treated total RNA. Primers and probes were designed using Primer Express SoftwareTM (Applied Biosystems, Foster CA). Target probe was labeled with fluorescent reporter dye FAM. Probe sequences are available upon request. Reporter dye emission was detected by an automated sequence detector combined with ABI Prism 7700 Sequence Detection System® software (Applied Biosystems). Real time PCR quantification was then performed using TaqMan® 18S controls.

FA analysis. Dietary lipids were extracted according to a modified Bligh and Dyer method (21). Ileal samples were homogenized in methanol with 0.05% BHT: chloroform (2:1, v:v). The homogenate lipids were extracted and loaded on thin layer chromatography plates (Silica gel G, 20x20, Analtech, Inc. DE). Plates were

developed half -way in hexane:diethyl-ether:acetic acid (40:60:1, v:v:v), allowed to dry, and re-developed entirely, in the same direction, with heptane:diethyl-ether:formic acid (80:20:1.8, v:v:v). The separated lipid class bands were visualized in iodine vapor and the PL fraction was collected. Fatty acid methyl esters (FAME) for dietary lipids and ileal PL were prepared using 14% BF3 in methanol (Sigma Chemical, St. Louis, MO).

FAME analyses were performed using a Hewlett Packard 5890 Gas Chromatography-Flame Ionization Detector (GC-FID). A BPX-70 column (25m × 0.22mm × 0.25μm, SGE, Austin, Tx) was used for the analysis with H_2 as the carrier gas. FAME identities were determined by electron impact mass spectrometry (MS), using a Varian Star 3400 GC coupled to a Varian Saturn 2000 ion trap MS. FAME identities were based on GC retention time of each substance and their electron impact mass spectra. An equal weight FAME mixture (68A; Nuchek Prep, Elysian, MN) was used to calculate response factors. The following pure BCFA were also used as a reference: *iso*-14:0, *anteiso*-15:0; *iso*-16:0, *anteiso*-17:0, *iso*-18:0 and *iso*-20:0 (Larodan Fine Chemicals AB, Malmo, Sweden). FA levels are expressed as %, weight for weight (%w/w).

***Statistics*.** The Chi-Square test was used to analyze differences in incidence of NEC. ANOVA followed by Fisher PLSD (StatView for Macintosh, Abacus Concepts, Inc., Berkely, CA) was used to analyze differences in cytokine gene expression. Tukey LSD, (JMP 8, SAS Institute, Cary, NC) was used to investigate differences in weights between the groups.

Results

The overall average survival rate was 89% (65/73). It was similar among the groups, with 87% (13/15), 89% (31/35), and 91% (21/23) in the DF, NEC and BCFA groups, respectively. Body weights at the end of study were 8.06 ± 0.24, 5.97 ± 0.05, and 5.69 ± 0.10g (mean ± SEM) for the DF, NEC and BCFA groups, respectively. There were no significant differences between the NEC and BCFA groups. The high relative weight of DF pups compared to the artificial feeding groups was similar to previous studies (20, 22, 23).

Figure 2.2 shows the histological NEC scores (**A**) and the incidence of NEC (**B**) in the experimental groups. Pups in the DF group do not normally develop NEC and no pup in the present study exhibited an abnormal intestinal architecture. In the NEC group, 17 of 31 (55%) of pups had a score of 2 or greater indicating NEC injury. In contrast, the BCFA group had histological scores of 2 or greater in 5 of 21 pups (24%). The difference in NEC development between NEC and BCFA groups was statistically significant ($p<0.05$), indicating that BCFA substituted for conventional fat reduced the incidence of NEC by 56% (55% vs 24%).

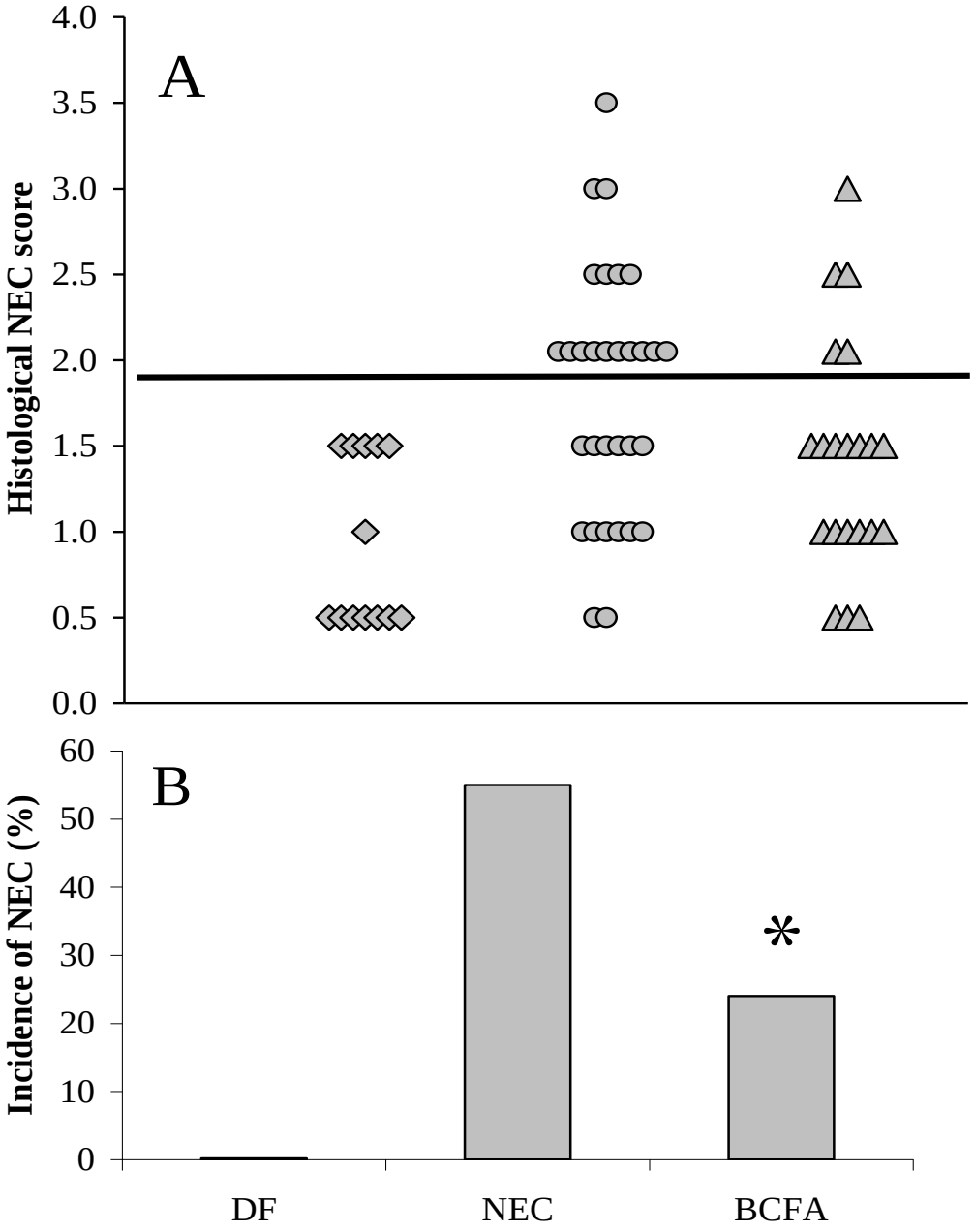

Figure 2.2. Histological scores (A) and incidence of necrotizing enterocolitis (NEC) (B) in the different treatment groups. DF- dam- fed pups, n=13; NEC- pups fed a rat milk substitute, n=31. BCFA- pups fed a rat milk substitute with BCFA, n=21. Animals were considered having NEC if their histological score was ≥ 2; *$p<0.05$.

***BCFA uptake by rat ileum*. Figure 2.3** presents BCFA concentrations (%w/w; mean ± SD) in ileum PL fraction of 3 pups without NEC from the BCFA group. BCFA were taken up by the ileum and were incorporated in the ileum PL. Furthermore, BCFA were incorporated in a selective manner in the PL: *iso*-14:0 and *anteiso*-15:0 were selected against and *iso*-18:0 was enriched in PL, compared to their proportions in the diet. Concentrations of BCFA in the ileum PL fraction of the NEC group were negligible (data not shown). The profile of other, non-branched FA in ileum PL was similar in the two hand-fed groups (data not shown).

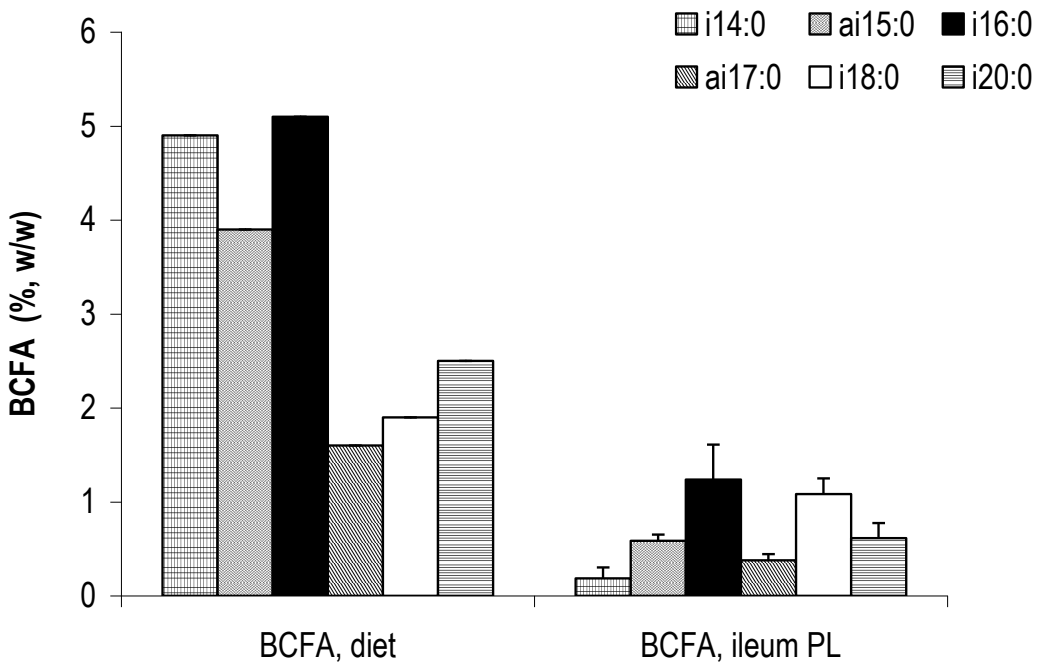

Figure 2.3. Branched chain fatty acid (BCFA) concentrations (%w/w) in BCFA diet and in ileum phospholipids (PL) of pups fed BCFA diet (n=3; mean ± SD). i=*iso*; ai=*anteiso*

Cytokine, mucin, TFF3 mRNA. **Table 2.2** presents results for selected mean ileal cytokine mRNA levels, expressed as multiples of the value found for the DF group. IL-10 levels were more than three-fold greater in the BCFA group compared to the DF and NEC groups. TGF-β levels in the BCFA and NEC groups were the same, and both were significantly lower than the DF group. IL-18 and TNF-α mRNA were significantly higher in the BCFA group compared to the DF group. IFN-γ levels in the BCFA group did not differ significantly from the DF group, but levels in the NEC group were significantly lower than the DF group.

Mean Muc2, Muc3, Muc4 and TFF3 mRNA levels, expressed as multiples of the value found for the DF group are also presented in **Table 2.2**. No differences in Muc3 and TFF3 mRNA among the experimental groups were observed; mRNA of the Muc2 and Muc4 mRNA were similar in the artificial feeding groups.

Table 2.2 Cytokine, mucin (Muc), and trefoil factor 3 (TFF 3) mRNA levels in ileum of neonatal rats (mean ± SEM)

	DF	NEC	BCFA
IL-10	1.0 ± 0.1	1.0 ± 0.1	3.2 ± 1.5*
TGF-β	1.0 ± 0.1	0.7 ± 0.1†	0.7 ± 0.1†
IL-18	1.0 ± 0.1	1.3 ± 0.1	1.7 ± 0.1†
TNF-α	1.0 ± 0.1	2.4 ± 0.8	2.7 ± 0.7†
IFN-γ	1.0 ± 0.3	0.4 ± 0.1†	0.7 ± 0.4
Muc 2	1.0 ± 0.1	0.5 ± 0.1†	0.4 ± 0.1†
Muc 3	1.0 ± 0.1	1.6 ± 0.3	1.2 ± 0.2
Muc 4	1.0 ± 0.1	0.3 ± 0.8†	0.1 ± 0.1†
TFF 3	1.0 ± 0.1	1.3 ± 0.2	1.6 ± 0.2

Mean steady-state mRNA levels for the dam-fed group (DF) were assigned a value of 1 and mean mRNA levels from pups fed rat milk substitute (NEC) or rat milk substitute with BCFA (BCFA) are expressed as fold changes relative to this value.
n=10-23 pups per treatment.
*$p<0.05$ vs. NEC and vs. DF
†$p<0.05$ vs. DF

Discussion

BCFA are constituents of the GI tract of normal, healthy, term newborns (6). The BCFA chain length distribution in vernix is from about C11 to C26, while that of meconium is from C16 to C26. Moreover, most BCFA entering the GI tract as amniotic fluid-suspended vernix disappear into the GI tract and do not appear in meconium. In the present study we show that oral administration of BCFA significantly reduces the incidence and severity of NEC in a neonatal rat model. Importantly, BCFA from the diet are taken up by the ileum *in vivo* and incorporated in a structure-selective manner into the PL fraction of the ileum. Selection against shorter chain BCFA (<16 carbons) for incorporation into the PL fraction is consistent with our previous observation on the systematic shift of BCFA distribution between human vernix and meconium (6). These results demonstrate that BCFA are not inert components of the GI tract or used exclusively for energy, but are selectively incorporated into membrane lipids mediated by enterocytes.

The reduction in NEC incidence is similar compared to most prophylactic treatments targeting NEC in experimental studies (24-26). We speculate that altered membrane composition may change enterocyte resistance to the as-yet ill-defined initiating events associated with NEC. Moreover, the entry and possible accumulation or turnover of BCFA could signal that normal parturition is approaching, in analogy to blood cortisol. This speculation is intriguing in part because amniotic fluid suspended lung surfactant phospholipids appear to have a role in vernix particle entry into the amniotic fluid (8).

Changes in selected mRNA levels suggest possible mechanisms of BCFA protection against NEC. There is no obvious overall trend in inflammatory cytokine mRNA with

and without BCFA treatment. A specific, notable change is the increase in ileal IL-10 mRNA caused by BCFA relative to the NEC group. Properties of IL-10 in intestinal disease apparently related to anti-inflammatory action have been reported. An IL-10 knockout mice spontaneously develops chronic enterocolitis throughout the entire intestinal tract (27); administration of IL-10 prevents the development of inflammatory bowel disease (IBD), and it was suggested that IL-10 treatment can counteract effectors involved in intestinal lesions in these animals (28). A similar effect was previously reported in studies with epidermal growth factor (EGF) and maternal milk in a rat NEC model (23, 29). Protective effect of EGF against NEC was associated with 3-fold increase in ileal mRNA IL-10 (29). Likewise, reduced NEC incidence in rat-milk-fed versus formula-fed pups was associated with more than doubled ileal IL-10 mRNA levels (23). Others showed that subcutaneous administration of recombinant human IL-10 to NEC-induced (hypoxia) neonate rats reduced the severity of microscopic ileal lesions compared to untreated rats (30). Taken together, elevated ileal IL-10 mRNA in the current study is consistent with the hypothesis that it mediates NEC reduction in the BCFA group, possibly by counteracting pro-inflammatory effects of elevated TNF-α and IL-18 mRNA.

Our and other laboratories have shown that proinflammatory cytokines are important factors in NEC pathogenesis (29, 31, 32). Caplan et al (33) found high plasma levels of TNF-α in infants with NEC, but no correlation was observed between TNF-α levels and severity of the disease. In the present study, we have found that ileal gene expression of TNF-α is significantly increased in the NEC group compared with healthy controls. However, the BCFA treatment of NEC did not affected TNF-α mRNA levels in the site on injury. Levels of IFN-γ mRNA were significantly lower in the NEC group compared to DF but the BCFA group was not different. The

relationship of IFN-γ in NEC is ambiguous. Elevated intestinal IFN-γ levels have been reported in NEC patients (34), however no significant differences in IFN-γ were found between formula-fed-hypoxic NEC rats and non-hypoxic formula-fed groups with mild intestine damage in a study similar to ours (35) and in separate studies ileal IFN-γ mRNA was not related to neonatal rat pups NEC development (31).

Another mechanism by which BCFA may also protect against NEC is by improving the intestinal barrier against pathogens. Intestinal mucins and TFF3 participate in host defense mechanisms against microbial invasion (18) . No differences in TFF3 mRNA among the study groups were observed, and levels of mucin mRNA were similar in the NEC and BCFA groups, providing no evidence for alterations in barrier function.

Alternatively, BCFA may protect against NEC by establishing balanced and diverse intestinal flora. Abnormal gut colonization is a risk factor for NEC (11, 12). The establishment of *bifidobacteria*, the predominant commensal strain in breast fed term infants, is delayed and less abundant in preterms (36). BCFA are prominent membrane components of many bacterial species (37), and many *bifidobacteria* include BCFA at various levels (0.6-24%w/w). *Iso*-14:0 is the second most abundant FA in *bifidobacterium breve*, with levels as high as 24.6%w/w (38). BCFA in the fetal and neonatal gut may influence flora development by favoring the growth of commensal organisms, and deserves attention in future studies.

In conclusion, BCFA reduce the incidence of NEC by 56% in a neonatal rat pup model. Of the various outcomes measured, 3-fold increased ileal levels of the anti-inflammatory IL-10 cytokine in the BCFA-fed group has been previously linked to reduced bowel disease and may have mediated the effect. We did not measure the

establishment of commensal flora, however previous work shows that supplementation with a probiotic mix induced production of IL-10 by intestinal mucosa of patients with acute pouchitis (39), and lactobacillus species prevented colitis in IL-10 gene deficient mice (40). Future research will examine how BCFA might reduce NEC by affecting diversity and composition of intestinal flora.

REFERENCES

1. Gibson RA, Kneebone GM 1981 Fatty acid composition of human colostrum and mature breast milk. Am J Clin Nutr 34:252-257.
2. Egge H, Murawski U, Ryhage R, Gyorgy P, Chatranon W, Zilliken F 1972 Minor constituents of human milk. IV. Analysis of the branched chain fatty acids. Chem Phys Lipids 8:42-55.
3. Corso G, Colavita C, Esposito M, Roma R, Napoli C, Zamparelli M, Ansanelli V 1995 Gaschromatography-mass spectrometry analysis of fatty acids in human milk from forty puerperae living in southern Italy. Riv Eur Sci Med Farmacol 17:215-219.
4. Nicolaides N, Ray T 1965 Skin Lipids. 3. Fatty Chains in Skin Lipids. The Use of Vernix Caseosa to Differentiate between Endogenous and Exogenous Components in Human Skin Surface Lipid. J Am Oil Chem Soc 42:702-707.
5. Nicolaides N, Fu HC, Ansari MN, Rice GR 1972 The fatty acids of wax esters and sterol esters from vernix caseosa and from human skin surface lipid. Lipids 7:506-517.
6. Ran-Ressler RR, Devapatla S, Lawrence P, Brenna JT 2008 Branched chain fatty acids are constituents of the normal healthy newborn gastrointestinal tract. Pediatr Res 64:605-609.
7. Rissmann R, Groenink HW, Weerheim AM, Hoath SB, Ponec M, Bouwstra JA 2006 New insights into ultrastructure, lipid composition and organization of vernix caseosa. J Invest Dermatol 126:1823-1833.

8. Narendran V, Wickett RR, Pickens WL, Hoath SB 2000 Interaction between pulmonary surfactant and vernix: a potential mechanism for induction of amniotic fluid turbidity. Pediatr Res 48:120-124.
9. Miettinen TA, Luukkainen T 1968 Gas-liquid chromatographic and mass spectrometric studies on sterols in vernix caseosa, amniotic fluid and meconium. Acta Chem Scand 22:2603-2612.
10. Lin PW, Stoll BJ 2006 Necrotising enterocolitis. Lancet 368:1271-1283.
11. Caplan MS, Jilling T 2001 New concepts in necrotizing enterocolitis. Curr Opin Pediatr 13:111-115.
12. Claud EC, Walker WA 2001 Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. FASEB J 15:1398-1403.
13. McGuire W, Anthony MY 2003 Donor human milk versus formula for preventing necrotising enterocolitis in preterm infants: systematic review. Arch Dis Child Fetal Neonatal Ed 88:F11-14.
14. Lucas A, Cole TJ 1990 Breast milk and neonatal necrotising enterocolitis. Lancet 336:1519-1523.
15. Beeby PJ, Jeffery H 1992 Risk factors for necrotising enterocolitis: the influence of gestational age. Arch Dis Child 67:432-435.
16. Dvorak B, McWilliam DL, Williams CS, Dominguez JA, Machen NW, McCuskey RS, Philipps AF 2000 Artificial formula induces precocious maturation of the small intestine of artificially reared suckling rats. J Pediatr Gastroenterol Nutr 31:162-169.
17. Baguma-Nibasheka M, Brenna JT, Nathanielsz PW 1999 Delay of preterm delivery in sheep by omega-3 long-chain polyunsaturates. Biol Reprod 60:698-701.

18. Khailova L, Dvorak K, Arganbright KM, Halpern MD, Kinouchi T, Yajima M, Dvorak B 2009 Bifidobacterium Bifidum Improves Intestinal Integrity in a Rat Model of Necrotizing Enterocolitis. Am J Physiol Gastrointest Liver Physiol.
19. Clark JA, Doelle SM, Halpern MD, Saunders TA, Holubec H, Dvorak K, Boitano SA, Dvorak B 2006 Intestinal barrier failure during experimental necrotizing enterocolitis: protective effect of EGF treatment. Am J Physiol Gastrointest Liver Physiol 291:G938-949.
20. Dvorak B, Halpern MD, Holubec H, Williams CS, McWilliam DL, Dominguez JA, Stepankova R, Payne CM, McCuskey RS 2002 Epidermal growth factor reduces the development of necrotizing enterocolitis in a neonatal rat model. Am J Physiol Gastrointest Liver Physiol 282:G156-164.
21. Bligh EG, Dyer WJ 1959 A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911-917.
22. Nadler EP, Dickinson E, Knisely A, Zhang XR, Boyle P, Beer-Stolz D, Watkins SC, Ford HR 2000 Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. J Surg Res 92:71-77.
23. Dvorak B, Halpern MD, Holubec H, Dvorakova K, Dominguez JA, Williams CS, Meza YG, Kozakova H, McCuskey RS 2003 Maternal milk reduces severity of necrotizing enterocolitis and increases intestinal IL-10 in a neonatal rat model. Pediatr Res 53:426-433.
24. Caplan MS, Miller-Catchpole R, Kaup S, Russell T, Lickerman M, Amer M, Xiao Y, Thomson R, Jr. 1999 Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Gastroenterology 117:577-583.
25. Siggers RH, Siggers J, Boye M, Thymann T, Molbak L, Leser T, Jensen BB, Sangild PT 2008 Early administration of probiotics alters bacterial colonization

and limits diet-induced gut dysfunction and severity of necrotizing enterocolitis in preterm pigs. J Nutr 138:1437-1444.

26. Lu J, Jilling T, Li D, Caplan MS 2007 Polyunsaturated fatty acid supplementation alters proinflammatory gene expression and reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Pediatr Res 61:427-432.

27. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W 1993 Interleukin-10-deficient mice develop chronic enterocolitis. Cell 75:263-274.

28. Rennick DM, Fort MM 2000 Lessons from genetically engineered animal models. XII. IL-10-deficient (IL-10(-/-) mice and intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 278:G829-833.

29. Halpern MD, Dominguez JA, Dvorakova K, Holubec H, Williams CS, Meza YG, Ruth MC, Dvorak B 2003 Ileal cytokine dysregulation in experimental necrotizing enterocolitis is reduced by epidermal growth factor. J Pediatr Gastroenterol Nutr 36:126-133.

30. Ozturk H, Dokucu AI, Ogun C, Buyukbayram H 2002 Protective effects of recombinant human interleukin-10 on intestines of hypoxia-induced necrotizing enterocolitis in immature rats. J Pediatr Surg 37:1330-1333.

31. Halpern MD, Holubec H, Dominguez JA, Williams CS, Meza YG, McWilliam DL, Payne CM, McCuskey RS, Besselsen DG, Dvorak B 2002 Up-regulation of IL-18 and IL-12 in the ileum of neonatal rats with necrotizing enterocolitis. Pediatr Res 51:733-739.

32. Sharma R, Tepas JJ, 3rd, Hudak ML, Mollitt DL, Wludyka PS, Teng RJ, Premachandra BR 2007 Neonatal gut barrier and multiple organ failure: role of endotoxin and proinflammatory cytokines in sepsis and necrotizing enterocolitis. J Pediatr Surg 42:454-461.

33. Caplan MS, Sun XM, Hseuh W, Hageman JR 1990 Role of platelet activating factor and tumor necrosis factor-alpha in neonatal necrotizing enterocolitis. J Pediatr 116:960-964.

34. Ford H, Watkins S, Reblock K, Rowe M 1997 The role of inflammatory cytokines and nitric oxide in the pathogenesis of necrotizing enterocolitis. J Pediatr Surg 32:275-282.

35. Liu Y, Zhu L, Fatheree NY, Liu X, Pacheco SE, Tatevian N, Rhoads JM 2009 Changes in intestinal Toll-like receptors and cytokines precede histological injury in a rat model of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 297:G442-450.

36. Sakata H, Yoshioka H, Fujita K 1985 Development of the intestinal flora in very low birth weight infants compared to normal full-term newborns. Eur J Pediatr 144:186-190.

37. Kaneda T 1991 Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol Rev 55:288-302.

38. Veerkamp JH 1971 Fatty acid composition of Bifidobacterium and Lactobacillus strains. J Bacteriol 108:861-867.

39. Ulisse S, Gionchetti P, D'Alo S, Russo FP, Pesce I, Ricci G, Rizzello F, Helwig U, Cifone MG, Campieri M, De Simone C 2001 Expression of cytokines, inducible nitric oxide synthase, and matrix metalloproteinases in pouchitis: effects of probiotic treatment. Am J Gastroenterol 96:2691-2699.

40. Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN 1999 Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology 116:1107-1114.

CHAPTER 3

BRANCHED CHAIN FATTY ACID COMPOSITION OF RETAIL COW'S MILK*

abstract

Milk and other dairy products contribute significant amounts of FA to the average American's overall daily FA intake. The fatty acid profile of milk gained attention due to (a) its overall saturated fat content, associated with coronary heart disease, and (b) individual fatty acids, such as conjugated linoleic acid, associated with potential health benefits. Branched chain fatty acids (BCFA), are a class of primarily saturated fatty acids with a methyl branch or more on the carbon chain. BCFA profiles and levels in cow's milk in response to different diets and supplementations have been reported. Their distribution in retail milk in the United States, however, has not been reported. BCFA are found in trace levels in non-ruminant tissues, but they are a major component in vernix. Vernix, suspended in amniotic fluid, is normally swallowed by the fetus, thus introducing BCFA into the newborn intestinal gut. This indicates that humans are exposed to BCFA from a very early age. BCFA are also found in human milk, continuing the supply of BCFA. Although BCFA from the *iso*- and *anteiso*- type were not investigated in relation to human health, evidence suggests a beneficial role of these BCFA in *in vitro* and *in vivo* models. As data on possible beneficial effects of BCFA on human health start to emerge, information on their concentrations in retail milk becomes valuable. Our aim was to investigate the profile and levels of BCFA in retail whole milk in the United States. Methods: Whole fluid milk samples, packed

* Ran-Ressler RR, Sim, D, O'Donnell AM, Bauman DE Branched chain fatty acids composition of retail cow's milk. Submitted to the Journal of Dairy Science, August 2010.

for transport to retail stores, were obtained from 56 processing plants across the contiguous 48 United States. Milk samples were conventionally produced. Milk samples were shipped on ice overnight to Cornell University and immediately processed for FA analysis. Fatty acids were extracted with conventional methods and were analyzed using gas chromatography coupled with mass spectrometry.
Results and discussion: Retail milk samples contain BCFA with 14-18 carbons, and all the BCFA were from the *iso*- and *anteiso*- configuration. BCFA with fewer than 14 carbons and multi-methyl BCFA such as phytanic acid and pristanic acid were not detected. Levels of the total BCFA were 2.05 ± 0.14 (%w/w; mean ± SD), and BCFA from the *anteiso*- configuration comprised more than half of the total BCFA. An estimation of BCFA consumption from retail milk was calculated. It was concluded that BCFA may contribute a substantial amount of the daily fat intake, in amounts exceeding intake of some of the n-3 long chain fatty acids.

Introduction

Milk and other dairy products contribute significant amounts of FA to the average American's overall daily FA intake. Cow's milk fat is characterized by relatively high short and medium chain (C12-C16) fatty acids, and low polyunsaturates. Individual fatty acids, such as conjugated linoleic acids (CLA), have a beneficial effect on health maintenance and prevention of acute and chronic disease (1-2). The fatty acid (FA) profile of milk in all species, including cow's milk, is sensitive to dietary fat intake, for instance, concentrate versus pasture versus fish oil supplementation (3-6), thus milk composition may differ depending on production practices. We recently reported the composition of major FA retail milk in the United States (US) (7). About 3 % of

FA were unidentified and listed as a group under “Other”; branched chain fatty acids (BCFA) are included in this group.

BCFA are primarily saturated FA (SFA) with one or more methyl branches. In human metabolism, they are best studied as components of skin lipids. Overall, several dozen specific BCFA, are found in milk and tissue of ruminants, including sheep and goats, presumably synthesized by ruminal organisms that rely on them for membrane lipids (8-9). BCFA function in the membranes similarly to *cis* unsaturated double bonds; both interfere with the ability of saturated hydrocarbon chains to pack tightly forming rigid extended structures. Old data show that an *E. coli* strain that lost the ability to desaturate saturated FA were restored to wild-type growth rates by the addition of BCFA (10). Many rumen microorganisms, such as *Ruminococcus Flavefaciens c1a* and *Fusobacterium sp 9-21* have membranes with high concentrations of BCFA, with levels up to 80% of membrane FA (11).

BCFA are conveniently categorized as mono- and di-/multi- methyl BCFA; in monomethyl BCFA, the predominant branching is at the terminal methyl (*iso*) or next to the terminal methyl (*anteiso*), as shown in **Figure 1.1**. *iso*- and *anteiso*-BCFA are the main BCFA reported in cow’s milk (3-4, 6, 12-13). Terpenoid BCFA, exemplified by internal periodic poly-methyl branching such as phytanic acid (3,7,11,14 tetra-methyl hexadecanoic acid) and its alpha oxidation product pristanic acid (2,6,10,14 tetra-methyl pentadecanoic acid) are also reported in cow’s milk (13-14).

Information on human intake and metabolism of BCFA is scant. We recently showed that BCFA are a major component of the late term fetal and newborn gut contents (15). They comprise almost one third of the FA in vernix (15), the white fatty film that covers the fetus in utero. Vernix suspended in amniotic fluid is normally

swallowed by the fetus, increasingly so as parturition approaches (16), exposing the fetal gut to BCFA from an early age. Moreover, BCFA are also found in human milk (17-18) where they reportedly comprise 1.5%w/w. A 1981 report put the concentrations of *anteiso*-17:0 in colostrum at 0.45%w/w, higher than the concentrations of docosahexaenoic acid (DHA; 22:6n-3; 0.32%w/w) and arachidonic acid (20:4n-6; 0.4%w/w) (18). A study in rat pups shows that BCFA reduce the incidence of necrotizing enterocolitis (NEC), a devastating intestinal disease affecting premature infants (Ran-Ressler et al, unpublished data). Thus, BCFA are a major component in perinatal nutrition, and although they may have a beneficial role in human health, their presence in the US food chain has been almost completely neglected.

In a 1994 survey, milk contributed 7.5% of the protein, 4.2% of the total fat, and approximately 8% of the total saturated fat in the diet of adults (19). Based on NHANES 1999-2004 (20) fluid milk, mostly whole milk, provided 7.5%, and 6.4% of energy intake in children age 2-4 and 5-10, respectively. We hypothesized that BCFA are found in retail milk in the US and thus are consumed in substantial amounts in the American diet. We report here the first data on the structure and quantitative analysis of BCFA in a representative sample of the US retail milk supply.

Methods

Sampling. Conventionally produced whole fluid milk samples were obtained from 56 milk processing plants across the contiguous 48 United States. All samples were obtained in December, 2008. All samples were homogenized, pasteurized and packaged for transport to retail stores. Processing plants were selected based on the

criteria that they represented at least 50% of the volume of milk produced in that area. Milk was shipped on ice overnight to Cornell University and immediately processed for the analysis of FA composition.

Fatty Acids Analysis. Milk fat extraction was based on the Mojonnier method (AOAC 995.19) as modified by Barbano (21). Briefly, milk fat was obtained from 10 ml whole fluid milk by a sequence of 3 successive extractions. Ten ml of 95% alcohol and 25 ml ethyl ether plus milk was followed by vigorous mixing, then 25 ml petroleum ether was added and followed by vigorous mixing, and decanting of the ether layer. The second and third extractions were similar, except the volume of solvents was reduced to 5 ml of 95% alcohol and 15 ml each of ethyl and petroleum ethers, and the third extraction omitted the 95% alcohol. Ether solutions from the 3 extractions were combined, dried and re-suspended in hexane. Methyl esters of the extracted fat were prepared using sodium methoxide as the methylation reagent, according to Christie (22) as modified by Chouinard (23).

Fatty acid methyl ester (FAME) analyses were performed using a Hewlett Packard 5890 Gas Chromatograph (GC) with flame ionization detector. A BPX-70 column (25m × 0.22mm × 0.25µm, SGE, Austin, TX) was used for the analysis with H_2 carrier gas. The oven program was initially 80°C for one minute, increased 30°C per minute to 170°C and held for two minutes, then increased by 10°C per minute until a final temperature of 240°C held for one minute.

FAME identities were determined by electron impact mass spectrometry **(**MS), using a Varian Star 3400 GC coupled to a Varian Saturn 2000 ion trap MS, based on GC retention times and electron impact mass spectra. An equal weight FAME mixture

(68A; Nuchek Prep, Elysian, MN) was used to calculate response factors. Several pure BCFA were also used as reference standards: *iso*-14:0, *anteiso*-15:0; *iso*-16:0, *anteiso*-17:0, *iso*-18:0 and *iso*-20:0 (Larodan Fine Chemicals AB, Malmo, Sweden). FA levels were expressed as weight % for all FA. Under these conditions, two pairs of FAME coelute, *iso*-15:0/14:1 and *iso*-17:0/16:1. GC with covalent adduct chemical ionization tandem mass spectrometry (CACI-MS/MS) was used to resolve the two sets of overlapping peaks (24). Solutions of pure 14:1, 15:0, 16:1, and 17:0 FAME (0.5 μg/μl, Nucheck Prep Inc, Elsyian, MN) were used to determine response factors for ions characteristic of the respective FAME. Selected characteristic ions were plotted to resolve and quantify co-eluting FAME. MH^+ was used for the *iso*-FAME in the milk sample, and the sum of $[MH-32]^+$, MH^+, and $[M+54]^+$ intensities were used applied to monounsaturated FAME. The relative percent contribution of the two interfering FAME was applied to the coeluting peaks to produce yield pure intensities.

Results and discussion

***BCFA in Retail Milk*. Figure 3.1** presents in summary form the major classes of FA found in the present retail milk samples. The sum of palmitic acid (16:0) and all other saturated FA (SFA) with shorter chain lengths comprised about 51%w/w (mean), saturated FA with 18 or more carbons were 12%. These values were within experimental error of those reported previously for our similar comprehensive retail milk sampling. Monounsaturated FA (MUFA) were about 30% and polyunsaturated FA (PUFA) were 4.7%, again within experimental error of previously reported composition.

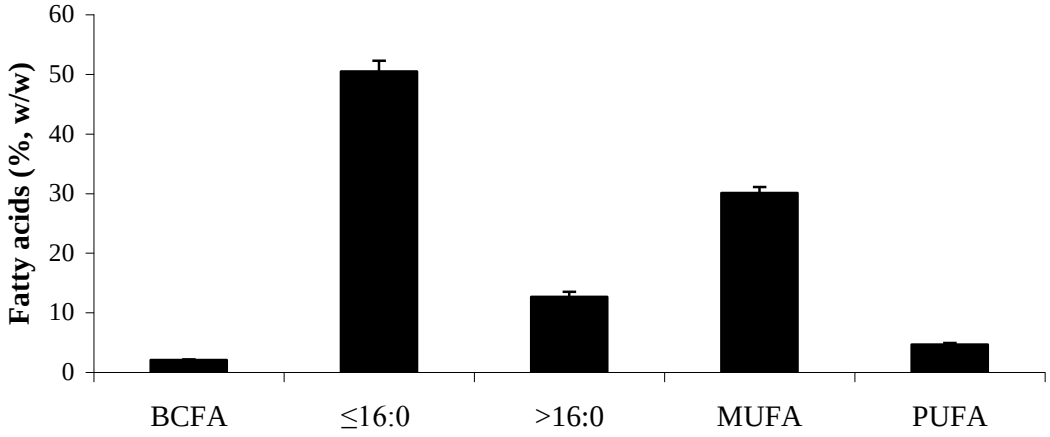

Figure 3.1. Fatty acid composition (%w/w; mean ± SD; n=56) of conventional whole milk samples. Fatty acids were grouped as follow: branched chain fatty acids (BCFA); *de-novo* synthesized saturated fatty acids, including 16:0 (≤ 16:0); saturated fatty acids longer then 16:0 (>16:0); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA).

BCFA comprised a total of 2.05 ± 0.14% of FA, or most of the 3% of FA listed previously as "other" (7). The mean BCFA levels reported in the present study fall within the range previously reported in studies that measured cow's milk FA in response to various diets in small scale experimental studies (3-5, 8, 12).

Table 3.1 presents the mean proportions of BCFA in retail milk as %w/w of total and as a % of total BCFA (Mean ± SD). BCFA with 14 to 18 carbons and *iso*- or *anteiso*-configuration were detected, and no BCFA with multiple branching were found. Four *iso*-C14 to *iso*-C17 were detected in all samples; *iso*-18:0 was also detected in most but not all of the samples. *anteiso*-15:0 and *anteiso*-17:0 were the only *anteiso* BCFA

found. They were in comparable concentration and constituted more than half of the total BCFA detected.

Table 3.1. Branched chain fatty acids (BCFA) in retail milk expressed as %w/w of total fatty acids and as % of BCFA (n=56; mean + SD).

BCFA	Total FA	BCFA
iso-14:0	0.13 ± 0.04	6.4 ± 1.4
iso-15:0	0.13 ± 0.01	6.6 ± 0.4
anteiso-15:0	0.56 ± 0.03	27.5 ± 1.3
iso-16:0	0.31 ± 0.03	14.9 ± 0.9
iso-17:0	0.26 ± 0.02	12.7 ± 0.7
anteiso-17:0	0.61 ± 0.06	29.9 ± 2.0
iso-18:0	0.04 ± 0.02	1.9 ± 0.9

BCFA with fewer than 14 carbons, such as *iso*- and *anteiso*- 13:0, were not detected in any of the samples. Both *iso*- and *anteiso*-13:0 BCFA in cow's milk have been reported by some (3, 5, 8, 13) but not by others (4, 6, 25). Retail milk is obtained from dairies that pool milk from many farms, and it is possible that specific feeds on some

farms support ruminal C13 BCFA more than on other farms, and thus small amounts may be diluted to below detection limits (12, 26).

Neither phytanic acid, a product of released phytol from chlorophyll by rumen bacteria (27), nor its peroxisomal oxidation product pristanic acid (28), were detected in the retail milk. As with C13 BCFA, these terpenoid BCFA were reported to be present in bovine milk by some (13-14) but not by others (3-6). When reported, the variance of these BCFA was high, and it was suggested (14) that differing feed compositions are responsible for variable terpenoid metabolizing ruminal bacteria. As with the case of *iso*- and *anteiso*- 13:0, it is possible that minor amounts of phytanic and pristanic acid were present, but diluted to below detection limits.

For comparison with bioactive FAs found primarily in dairy products, rumenic acid (*cis*-9, *trans*-11-18:2), the most concentrated conjugated linoleic acid (CLA) in cow's milk, was 0.57%, in conventionally produced retail milk. Vaccenic acid (*trans*-11-18:1), the most concentrated trans monoene in cow's milk, was 1.45% in U.S. retail milk, and the sum of all trans FA was about 3% (7). Thus, both *anteiso* BCFA are at greater concentration in milk fat than rumenic acid, and total BCFA is more than half the concentration of total *trans*- FA.

Estimated human BCFA intake from retail milk. We can estimate the contribution of BCFA to the nutrition of Americans based on measured and estimated intake of milk. One cup (244 g = 8 oz) of whole milk (3.25% milkfat) contains 7.9 g fat (29), 2% of 7.9 g yields a total of 158 mg BCFA per cup whole milk. For comparison to intake of bioactive FA in the American diet, this value is greater than the 100 mg average *daily* consumption of the DHA and eicosapentaenoic acid (EPA) reported in a survey of

8604 Americans between 1999 and 2000 (30) and by women of child bearing age based on NHANES III data (31).

In some population groups, such as small children, milk consumption can be higher: consumption by small children of 2.6 servings of milk was reported recently (32); most of the children in the study consumed whole milk. This consumption would thus provide 412mg BCFA daily, which would provide almost 1% of the total fat intake of children age 2-5 (33). It thus appears that the absolute amount of daily intake of BCFA from milk--even more so, the amount of BCFA consumed per kilogram body weight--will be higher in some populations, such as in small children.

Apart from milk, other foods produced by ruminant animals, specifically cheese and beef, are expected to be other primary contributors of BCFA to the diet. US cheese BCFA can be estimated from the present measures of milkfat BCFA of about 2% of FA. BCFA averaged about 2%, w/w in Canadian retail beef (34), and similar to Malaysia beef tallow with C13-C20 BCFA with mean concentration 2.3% (mutton tallow had 4.0% BCFA) (35). We assume American beef has the same 2% BCFA levels as in Canada, and an average 28% and about 18% fat content in cheese and in beef, respectively (29). According to economic disappearance data adjusted for loss, American's average per capita consumption of cheese is 30 g (1.1 oz) per day (36); at about 28% fat on average and at about 2% BCFA, cheese contributes 168 mg of BCFA per day. Similarly, Americans consume 50 g (1.8 oz) of cooked beef per day (36); at about 18% fat and about 2% BCFA, beef contributes about 180 mg of BCFA per day. **Table 3.2** presents these values summed to estimate total current per capita intake of BCFA of about 400 mg per day from the most common ruminant foods.

We also consider the implications of dietary guidelines on BCFA consumption (37). Americans are advised to consume three servings from the milk group, where a serving, for example, is equal to 1 cup of milk or yogurt and 1.5 oz (42 g, 2 slices) of cheese. For simplicity we assume this to be represented by the present average dietary pattern of milk intake in the US, which is 31% whole milk, 39% low fat (2%) milk, 14% lower fat (1%) milk, with the balance nonfat milk (36). Using these values we estimate a weighted sum of the total BCFA intake from three servings of milk and cheese to be nearly 400 mg. Meat, cheese and milk together account for an average estimated intake of about 575 mg BCFA per capita per day (**Table 3.2).**

Table 3.2. Estimated mean daily branched chain fatty acids consumption per capita in the US[1], based on actual and recommended[2] consumption of milk, cheese and beef

	Actual[2]		Recommended[2]	
Food	Portion (g)	BCFA[3] (mg)	Portion (g)	BCFA[3] (mg)
Milk[4]	119	54	215	98
Cheese[5]	30	168	53	297
Beef[6]	50	180	50	180
Total	199	402	318	575

[1]Intake is based on (36) [2]Actual consumption reflects the current consumption from milk, cheese and beef by Americans, which falls below recommended levels from the milk food group (36). Recommended consumption represents the levels of BCFA consumption that would obtain if Americans were to consume recommended amounts from the milk food group, keeping the same existing patterns as the actual consumption. For beef, the actual consumption was considered as the recommended one. Thus, the same value for beef was used for both. [3] BCFA - branched chain fatty acids

[4] Mean, per capita, milk consumption, based on the proportions currently consumed by Americans of 3.25%, 2%, and 1% milk (36) [5]Cheese types are the main cheeses consumed by Americans (36). An average of 28% fat was used for estimation (29). Amounts are based on patterns of current consumption of cheese (36). [6] For cooked beef, an average of 18% fat was used for estimation (29). Americans consume enough from the meat food group, thus the actual and the recommended consumption were the same.

Use of 2 cups of whole milk in place of reduced or no fat milks and consuming 1 serving of cheese (42gr, 1.5oz) would bring total BCFA to 731 mg/day (316 mg from milk + 235 mg from cheese + 180 mg from beef). These consumption levels, then, would be higher than the 500mg/d intake for DHA and EPA for the general population recommended by the American Dietetic Association (38), and more than double the 300 mg/d of DHA and EPA recommended by the World Health Organization in pregnancy and lactation (31).

These estimates show that daily BCFA intake is substantial and that current recommendations promote an increase in present intake, considerably high than other bioactive FA. Unlike DHA and EPA, for instance, BCFA sources include a greater variety of common food items, principally products of ruminants, regularly consumed by non-vegans. The low level of interest in BCFA nutrition is remarkable considering their long-standing intake.

The American dietary guidelines recommend consumption of low fat dairy products, however studies provide no convincing evidence that increased whole milk consumption is harmful with respect to ischemic heart disease and ischemic strokes (39). Some studies provide evidence that higher consumption of whole fat compared to reduced fat milk was associated with lower body mass index in preschool- and elementary school-age children and less weight gain in adults (32, 40-41) and with lower incidences of anovulatory infertility (42). Thus, the emergence of potential benefits of whole milk consumption may have a significant effect on BCFA intake in the US population, increasing BCFA consumption even more.

BCFA are synthesized in large amounts by human skin (44), comprise 29%w/w, of the FA in vernix and are normal constituents of the healthy newborn gut (15). A systematic shift in BCFA profile was observed between vernix and meconium of the same infant, implying that the fetal alimentary canal selectively metabolizes BCFA. In very recent work, anoxic rat pups fed a mixture similar to most BCFA found in the retail milk samples (*iso*-14:0, *anteiso*-15:0, *iso*-16:0, *anteiso*-17:0, *iso*-18:0, and *iso*-20:0) in rat milk substitute, had reduced the incidence of NEC compared to a control group (Ran-Ressler et al, unpublished data) and elevated mRNA levels of the intestinal, anti-inflammatory cytokine IL-10. BCFA are selectively incorporated into phospholipids of rat pup ileum and into human Caco-2 cells (45). The risk reduction in NEC development may be linked to these observations. Apoptotic properties of BCFA on human breast cancer cells are structure-specific, with *iso*-16:0 having the highest activity among BCFA of C12-C20 (46). *iso*-15:0 inhibited tumor growth in cultured cells and in an *in vivo* (mice) models with no obvious deleterious effects (47). Thus, BCFA of the type found in U.S. retail milk may have a beneficial effect on proper tissue function and on gut development and function.

In conclusion, we document for the first time the profile and amounts of BCFA in retail whole milk in the U.S. Milk BCFA have chain lengths of 14 to 18 carbons and include both *iso*- and *anteiso*- BCFA. BCFA comprising 2.05%w/w of the FA in retail whole milk, and along with estimated BCFA levels in beef and estimated per capita intakes can amount to a levels higher than many bioactive FA. BCFA are readily available from food consumed by healthy individuals in the US. Their importance in the U.S. food supply and bioactivity suggest that they should be more carefully studied for their biological effects.

REFERENCES

1. Lock AL, Bauman DE 2004 Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids 39:1197-1206.
2. Bauman DE LA, Corl BA, Ip C, Salter AM, Parodi PW. 2006 Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress. . Wageningen Academic Publishers, Wageningen, The Netherlands.
3. Boeckaert C, Vlaeminck B, Dijkstra J, Issa-Zacharia A, Van Nespen T, Van Straalen W, Fievez V 2008 Effect of dietary starch or micro algae supplementation on rumen fermentation and milk fatty acid composition of dairy cows. J Dairy Sci 91:4714-4727.
4. Craninx M, Steen A, Van Laar H, Van Nespen T, Martin-Tereso J, De Baets B, Fievez V 2008 Effect of lactation stage on the odd- and branched-chain milk fatty acids of dairy cattle under grazing and indoor conditions. J Dairy Sci 91:2662-2677.
5. Osborne VR, Radhakrishnan S, Odongo NE, Hill AR, McBride BW 2008 Effects of supplementing fish oil in the drinking water of dairy cows on production performance and milk fatty acid composition. J Anim Sci 86:720-729.
6. Chilliard Y, Martin C, Rouel J, Doreau M 2009 Milk fatty acids in dairy cows fed whole crude linseed, extruded linseed, or linseed oil, and their relationship with methane output. J Dairy Sci 92:5199-5211.
7. O'Donnell AM, Spatny KP, Vicini JL, Bauman DE 2010 Survey of the fatty acid composition of retail milk differing in label claims based on production management practices. J Dairy Sci 93:1918-1925.

8. Massart-Leen AM, DePooter H, Decloedt M, Schamp N 1981 Composition and variability of the branched-chain fatty acid fraction in the milk of goats and cows. Lipids 16:286-292.
9. Duncan WR, Garton GA 1978 Differences in the proportions of branched-chain fatty acids in subcutaneous triacylglycerols of barley-fed ruminants. Br J Nutr 40:29-33.
10. Silbert DF, Ladenson RC, Honegger JL 1973 The unsaturated fatty acid requirement in Escherichia coli. Temperature dependence and total replacement by branched-chain fatty acids. Biochim Biophys Acta 311:349-361.
11. Ifkovits RW, Ragheb HS 1968 Cellular fatty acid composition and identification of rumen bacteria. Appl Microbiol 16:1406-1413.
12. Vlaeminck B, Fievez V, Cabrita ARJ, Fonseca AJM, Dewhurst RJ 2006 Factors affecting odd- and branched-chain fatty acids in milk: A review. Animal Feed Science and Technology 131:389-417.
13. Vlaeminck B, Lourenco M, Bruinenberg M, Demeyer D, Fievez V 2004 Odd and branched chain fatty acids in rumen contents and milk of dairy cows fed forages from semi-natural grasslands. Commun Agric Appl Biol Sci 69:337-340.
14. Lough AK 1973 The chemistry and biochemistry of phytanic, pristanic and related acids. Prog Chem Fats Other Lipids 14:1-48.
15. Ran-Ressler RR, Devapatla S, Lawrence P, Brenna JT 2008 Branched chain fatty acids are constituents of the normal healthy newborn gastrointestinal tract. Pediatr Res 64:605-609.

16. Narendran V, Wickett RR, Pickens WL, Hoath SB 2000 Interaction between pulmonary surfactant and vernix: a potential mechanism for induction of amniotic fluid turbidity. Pediatr Res 48:120-124.
17. Egge H, Murawski U, Ryhage R, Gyorgy P, Chatranon W, Zilliken F 1972 Minor constituents of human milk. IV. Analysis of the branched chain fatty acids. Chem Phys Lipids 8:42-55.
18. Gibson RA, Kneebone GM 1981 Fatty acid composition of human colostrum and mature breast milk. Am J Clin Nutr 34:252-257.
19. Cotton PA, Subar AF, Friday JE, Cook A 2004 Dietary sources of nutrients among US adults, 1994 to 1996. J Am Diet Assoc 104:921-930.
20. Wiley AS 2010 Dairy and milk consumption and child growth: Is BMI involved? An analysis of NHANES 1999-2004. Am J Hum Biol 22:517-525.
21. Barbano DM, Clark JL, Dunham CE 1988 Comparison of Babcock and ether extraction methods for determination of fat content of milk: collaborative study. J Assoc Off Anal Chem 71:898-914.
22. Christie WW 1982 A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. Journal of Lipid Research 23:1072-1075.
23. Chouinard PY, Corneau L, Barbano DM, Metzger LE, Bauman DE 1999 Conjugated linoleic acids alter milk fatty acid composition and inhibit milk fat secretion in dairy cows. J Nutr 129:1579-1584.
24. Van Pelt CK, Brenna JT 1999 Acetonitrile chemical ionization tandem mass spectrometry to locate double bonds in polyunsaturated fatty acid methyl esters. Analytical Chemistry 71:1981-1989.
25. Vlaeminck B, Fievez V, van Laar H, Demeyer D 2004 Rumen odd and branched chain fatty acids in relation to in vitro rumen volatile fatty acid

productions and dietary characteristics of incubated substrates. J Anim Physiol Anim Nutr (Berl) 88:401-411.

26. Keeney M, Katz I, Allison MJ 1962 On Probable Origin of Some Milk Fat Acids in Rumen Microbial Lipids. Journal of the American Oil Chemists Society 39:198-&.

27. Patton S, Benson AA 1966 Phytol metabolism in the bovine. Biochim Biophys Acta 125:22-32.

28. Verhoeven NM, Wanders RJA, Poll-The BT, Saudubray JM, Jakobs C 1998 The metabolism of phytanic acid and pristanic acid in man: A review. Journal of Inherited Metabolic Disease 21:697-728.

29. USDA ARS 2009 USDA national nutrient database for standard reference, release 22. Nutrient data laboratory home page http://www.ars.usda.gov/ba/bhnrc/ndl August 3 2010

30. Ervin RB, Wright JD, Wang CY, Kennedy-Stephenson J 2004 Dietary intake of fats and fatty acids for the United States population: 1999-2000. Adv Data:1-6.

31. Brenna JT, Lapillonne A 2009 Background paper on fat and fatty acid requirements during pregnancy and lactation. Ann Nutr Metab 55:97-122.

32. Huh SY, Rifas-Shiman SL, Rich-Edwards JW, Taveras EM, Gillman MW 2010 Prospective association between milk intake and adiposity in preschool-aged children. J Am Diet Assoc 110:563-570.

33. USDA ARS 2010 Nutrient intakes from food: Mean amounts consumed per individual, by gender and age, *What we eat in America*, NHANES, 2007-2008. www.ars.usda.gov/ba/bhnrc.fsrg August 4 2010

34. Aldai N, Dugan MER, Kramer JKG 2010 Can the trans-18:1 and conjugated linoleic acid profiles in retail ground beef be healthier than steak? Journal of Food Composition and Analysis 23:326-332.

35. Chin ST, Man YBC, Tan CP, Hashim DM 2009 Rapid Profiling of Animal-Derived Fatty Acids Using Fast GC x GC Coupled to Time-of-Flight Mass Spectrometry. Journal of the American Oil Chemists Society 86:949-958.

36. USDA ERS 2010 Food availability (per capita) data system http://www.ers.usda.gov/Data/FoodConsumption July 30 2010

37. USDA 2005 Food guide pyramid http://www.mypyramid.gov/pyramid/index.html July 22 2010

38. Kris-Etherton PM, Innis S, Ammerican Dietetic A, Dietitians of C 2007 Position of the American Dietetic Association and Dietitians of Canada: dietary fatty acids. J Am Diet Assoc 107:1599-1611.

39. Elwood PC, Pickering JE, Hughes J, Fehily AM, Ness AR 2004 Milk drinking, ischaemic heart disease and ischaemic stroke II. Evidence from cohort studies. Eur J Clin Nutr 58:718-724.

40. Barba G, Troiano E, Russo P, Venezia A, Siani A 2005 Inverse association between body mass and frequency of milk consumption in children. Br J Nutr 93:15-19.

41. Rosell M, Hakansson NN, Wolk A 2006 Association between dairy food consumption and weight change over 9 y in 19,352 perimenopausal women. Am J Clin Nutr 84:1481-1488.

42. Chavarro JE, Rich-Edwards JW, Rosner B, Willett WC 2007 A prospective study of dairy foods intake and anovulatory infertility. Hum Reprod 22:1340-1347.

43. Kaneda T 1991 Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol Rev 55:288-302.

44. Nicolaides N, Ray T 1965 Skin Lipids. 3. Fatty Chains in Skin Lipids. The Use of Vernix Caseosa to Differentiate between Endogenous and Exogenous Components in Human Skin Surface Lipid. J Am Oil Chem Soc 42:702-707.

45. Ran-Ressler RR, Kothapalli KS, Glahn R, Brenna JT 2010 Branched Chain Fatty Acids are taken up and metabolized by Cultured Human Intestinal Cells (Caco-2). Experimental Biology, Anahein, Ca.

46. Wongtangtintharn S, Oku H, Iwasaki H, Toda T 2004 Effect of branched-chain fatty acids on fatty acid biosynthesis of human breast cancer cells. J Nutr Sci Vitaminol (Tokyo) 50:137-143.

47. Yang Z, Liu S, Chen X, Chen H, Huang M, Zheng J 2000 Induction of apoptotic cell death and in vivo growth inhibition of human cancer cells by a saturated branched-chain fatty acid, 13-methyltetradecanoic acid. Cancer Res 60:505-509.