



Published in final edited form as:

Pediatr Res. 2008 December ; 64(6): 605–609. doi:10.1203/PDR.0b013e318184d2e6.

Branched Chain Fatty Acids Are Constituents of the Normal Healthy Newborn Gastrointestinal Tract

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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. 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 was 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.

Keywords

branched chain fatty acids; vernix; meconium

Branched chain fatty acids (BCFA) are mostly saturated fatty acids (SFA) with one or more methyl branches on the carbon chain. 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.

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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% BF_3 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 H₂ 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. 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.

Overall FA distribution

A profile of FA classes is shown in Table 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.

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 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*.

The profile of the *iso* BCFA in vernix and meconium is shown in Figure 2. 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.

The profile of *anteiso* BCFA is shown in Figure 3. 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 4 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.

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 FA 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 $30 \times 6 = 180$ 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 has 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 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 *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 Nicolaidis & 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.

Acknowledgements

Financial Support: This work was supported by NIH grant GM071534

Abbreviations

BCFA, branched-chain fatty acids
 GC, gas chromatography
 GI, gastrointestinal
 FA, fatty acids
 FAME, fatty acid methyl esters
 MS, mass spectrometry
 MUFA, monounsaturated fatty acids
 SE, sterol esters
 SFA, saturated fatty acids
 TAG, triacylglycerol
 WE, wax esters

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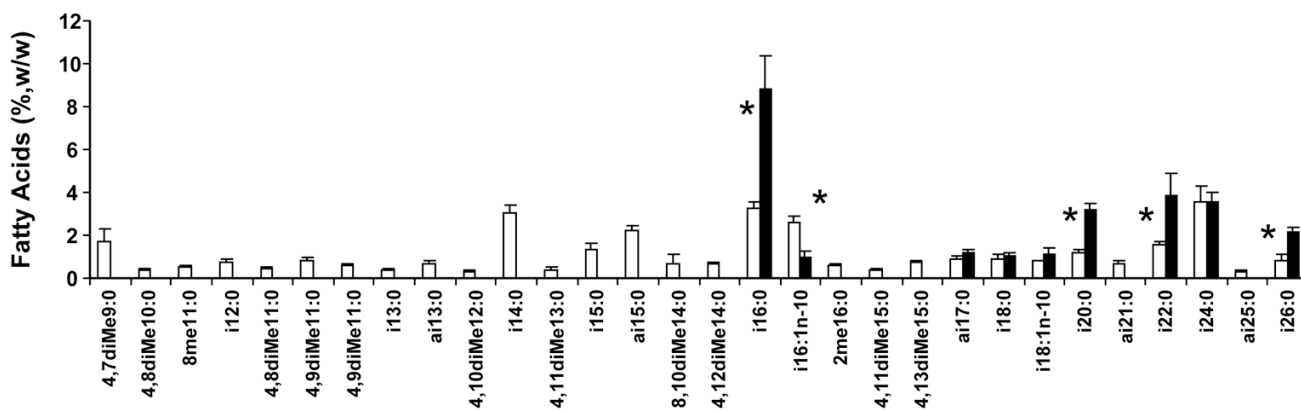


Figure 1.

BCFA methyl ester profiles for vernix and meconium (means \pm SEM, n \leq 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-methylhexadecanoic acid). *i*=*iso*; *ai*=*anteiso*; Me=methyl; diMe = two methyl branches. Key: \square vernix; \blacksquare meconium. *p<0.05

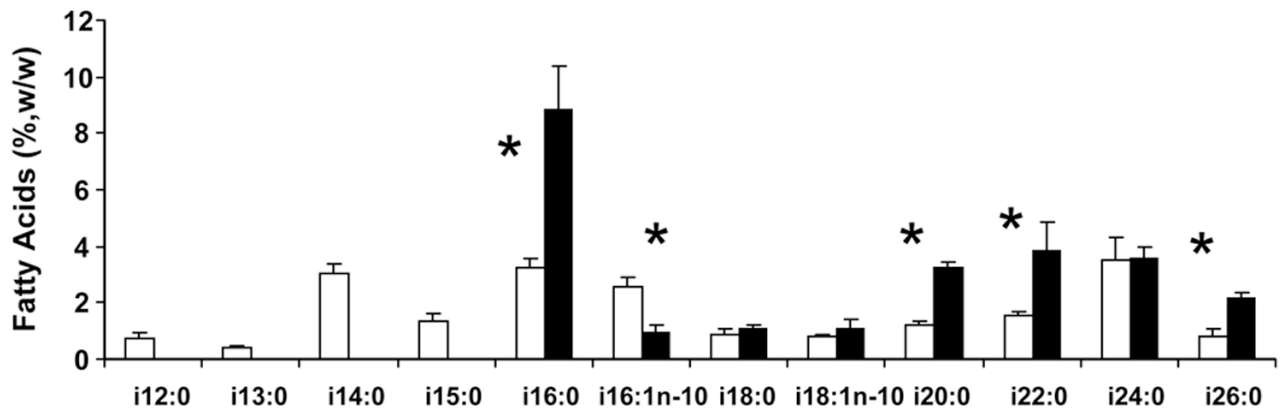


Figure 2. *Iso* BCFA methyl esters in vernix and meconium (means±SEM, n≤18 newborn) listed left to right in order of molecular weight. *i=iso*. Key: □ vernix; ■ meconium. *p<0.05

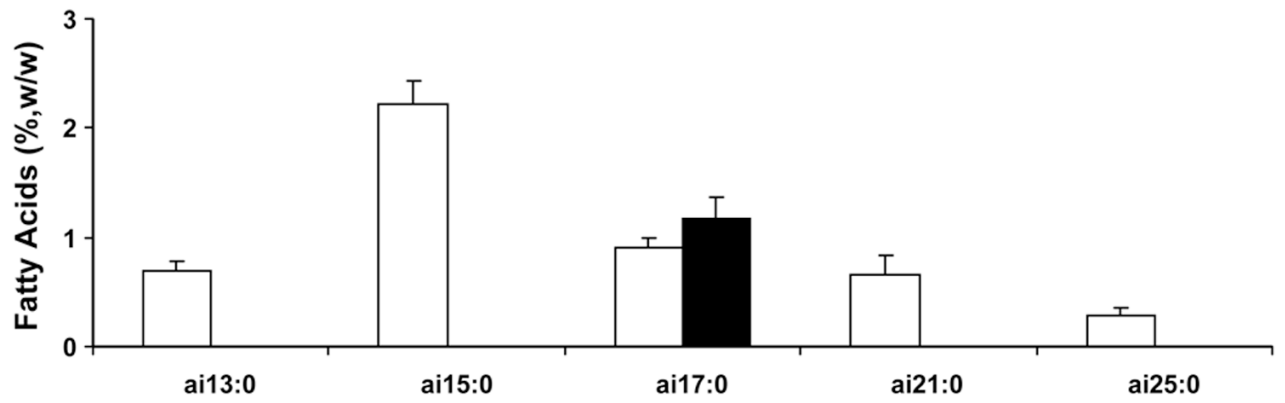


Figure 3. *Anteiso* BCFA methyl esters in vernix and meconium (means \pm SEM, n \leq 18 newborn) listed left to right in order of molecular weight. *ai*=*anteiso*. Key: \square vernix; \blacksquare meconium. *p<0.05

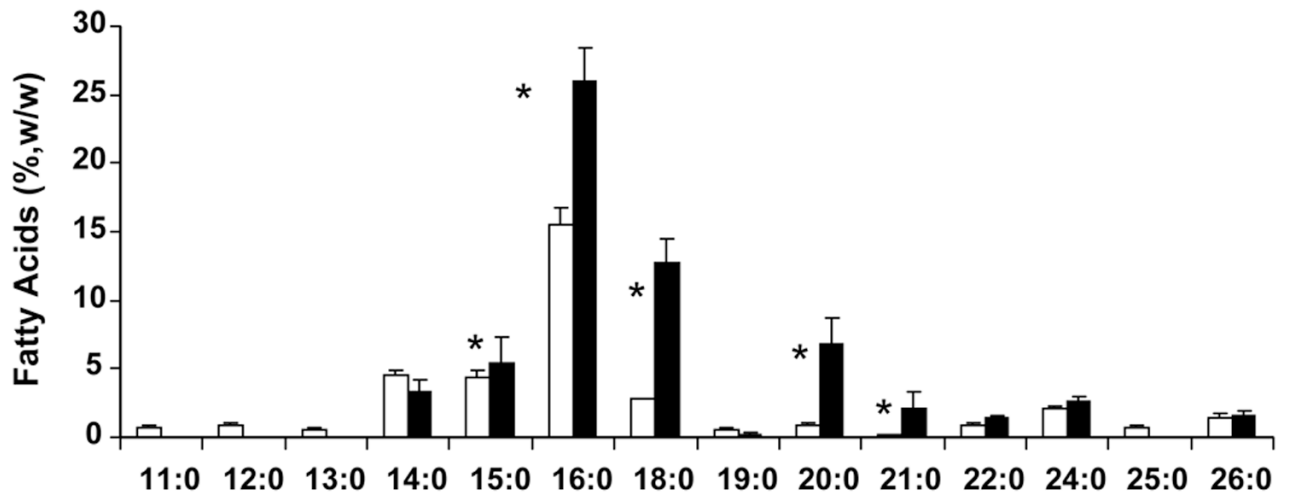


Figure 4. Normal (straight chain) FAME in vernix (means \pm SEM, $n\leq 18$ newborn) listed left to right in order of molecular weight. Key: \square vernix; \blacksquare meconium. * $p<0.05$.

Table 1

Characteristics of study population (mean±SD (range)).

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

* Cesarean section

Table 2

Profile of FA classes (%w/w) in vernix and meconium (mean±SEM)

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

*
p<0.05