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Milk-Derived Amadori Products in Feces of Formula-Fed Infants

Nina Sillner,[†,‡] Alesia Walker,*[,†,⊥] Daniel Hemmler,[†,§] Monika Bazanella,[∥] Silke S. Heinzmann,[†] Dirk Haller,[‡,∥] and Philippe Schmitt-Kopplin[†,‡,§]

[†]Research Unit Analytical BioGeoChemistry, Helmholtz Zentrum München, 85764 Neuherberg, Germany

[‡]ZIEL Institute for Food and Health, Technical University of Munich, 85354 Freising, Germany

[§]Chair of Analytical Food Chemistry, Technical University of Munich, 85354 Freising, Germany

[∥]Chair of Nutrition and Immunology, Technical University of Munich, 85354 Freising, Germany

Supporting Information

ABSTRACT: Food processing of infant formula alters chemical structures, including the formation of Maillard reaction products between proteins and sugars. We detected early Maillard reaction products, so-called Amadori products, in stool samples of formula-fed infants. In total, four Amadori products (*N*-deoxylactulosyllysine, *N*-deoxyfructosyllysine, *N*-deoxylactulosylleucylisoleucine, *N*-deoxyfructosylleucylisoleucine) were identified by a combination of complementary nontargeted and targeted metabolomics approaches. Chemical structures were confirmed by preparation and isolation of reference compounds, LC-MS/MS, and NMR. The leucylisoleucine Amadori compounds, which most likely originate from *β*-lactoglobulin, were excreted throughout the first year of life in feces of formula-fed infants but were absent in feces of breastfed infants. Despite high inter- and intraindividual differences of Amadori products in the infants' stool, solid food introduction resulted in a continuous decrease, proving infant formula as the major source of the excreted Amadori products.

KEYWORDS: *Amadori products, Maillard reaction, metabolomics, infant formula, milk marker, fructosyllysine*

INTRODUCTION

In the first months of life the only source of food for infants is breast milk or infant formula. During infant formula production and subsequent storage, Maillard reaction products are formed. For example, Amadori products, such as *ε*-*N*-deoxylactulosyllysine (LacLys), originate from lactosylation of 5−20% of the protein-bound lysine in infant formula.[1] In comparison, the glycation of protein-bound lysine in human breast milk is much lower however, depending on maternal nutrition. Under acidic conditions LacLys is partially released from milk proteins in the form of furosine, which is often used as an indicator of the degree of lysine glycosylation.[2,3] Clawin-Rädecker and Schlimme reported about 20−25 mg furosine/100 g of protein in breast milk from women in Germany.[4] Very low or nondetectable (below 6 mg furosine/100 g of protein) contents were measured in breast milk from women in Poland. But, in infant formula concentrations from 1320 to 1550 mg furosine/100 g of protein were detected.[5] However, the nutritional consequences for formula-fed infants are not completely understood. Studies have shown that *ε*-*N*-deoxyfructosyllysine (FruLys) was not available as a lysine source in vivo; therefore, a significant loss of nutritional value was suggested.[6] This loss is normally compensated by an increased protein amount in formula.[1] Also the bioavailability and excretion of milk-derived Amadori products ingested by formula-fed infants are largely unknown. In different intervention studies with adults or rats only 3−10% of peptide-bound Amadori products were absorbed and rapidly excreted via urine, whereas most of the Amadori products were suggested to be fermented by the gut microbiota.[7−9] In in vitro tests, FruLys was not actively transported by intestinal cells but rather absorbed by diffusion.[10,11] Different glycated dipeptides, however, might be absorbed and cross the basolateral membrane after amino acid hydrolysis inside colonic cells.[12]

Occasionally, novel food markers can be identified in nutrition studies by nontargeted metabolomics profiling without prior focusing on ingredients or metabolites.[13−15] In the present work, we conducted a multistep analytical approach to discover and identify milk-derived Amadori products in feces of formula-fed infants. This was achieved by comparing fecal samples from formula-fed and breastfed infants at several time points during their first year of life using a combination of liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy.

MATERIALS AND METHODS

Study Design. Stool samples (n = 244, Table S1) from healthy infants, who received infant formula with (n = 11, F+) or without (n = 11, F−) bifidobacteria (*B. bifidum*, *B. breve*, *B. infantis*, *B. longum*) or exclusively breast milk (n = 20, B), were collected over a period of two years in a randomized, double-blinded, placebo-controlled intervention trial as described elsewhere.[16] For metabolomics analysis, fecal samples of months 1, 3, 5, 7, 9, 12, and 24 were selected. The trial was registered at the German Clinical Trials Register under number DRKS00003660, and the protocol was approved by the ethics committee of the medical faculty of the Technical University of Munich (approval no. 5324/12).

Chemicals. L-Lysine, D-(+)-glucose, *α*-lactose monohydrate, and trimethylsilylpropionate (TSP; 98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Leucylisoleucine was purchased from Bachem (Bubendorf, Switzerland), and *ε*-*N*-deoxyfructosyllysine

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(dihydrochloride) was purchased from Abcam (Cambridge, U.K.). Milli-Q water (18.2 MΩ) was derived from a Milli-Q Integral Water Purifcation System (Billerica, MA, USA). Acetonitrile (ACN; LiChrosolv, hypergrade for LC-MS), methanol (LiChrosolv, hypergrade for LC-MS), and ammonium acetate (NH_4Ac) were obtained from Merck (Darmstadt, Germany). Glacial acetic acid was purchased from Biosolve (Valkenswaard, Netherlands).

Fecal Sample Preparation. Metabolite extraction from infant stool samples was prepared with methanol as described previously.[16] For direct infusion Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) analysis, the extracts were diluted 1:1000 (v/v) with methanol. For ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) measurements, the methanol extracts were evaporated under vacuum at 40 °C (SpeedVac Concentrator, Savant SPD121P, ThermoFisher Scientific, Waltham, MA, USA) and reconstituted with ACN/H_2O 75:25 (v/v). A pooled sample was generated from all fecal extracts for quality control purposes. All samples were stored at −80 °C in tightly closed tubes.

Direct Infusion FT-ICR Mass Spectrometry. Ultrahigh-resolution FT-ICR mass spectra of diluted fecal extracts from 1-month-old infants were acquired with a 12 T Bruker Solarix mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an APOLLO II electrospray source in negative ionization mode. The diluted samples were infused into the electrospray ion source with a flow rate of 2 μL/min. Settings for the ion source were a drying gas temperature of 180 °C, a drying gas flow of 4 L/min, and a capillary voltage of 3600 V. The spectra were acquired with a time-domain of 4 megawords, and 150 scans were accumulated within a mass range of 123−1000 Da. The mass spectrometer was first externally calibrated by ion clusters of arginine (57 nmol/mL in methanol). Internal calibration of each spectrum was conducted with a reference list containing omnipresent fatty acids. Raw spectra were post-processed by Compass DataAnalysis 4.2 (Bruker Daltonics, Bremen, Germany), and mass signals with a signal-to-noise ratio of at least 6 were exported to mass lists. All exported features were aligned into a matrix containing averaged mass signals values (maximum peak alignment window width: ± 0.5 ppm) and corresponding intensities of all analyzed samples.[17] FT-ICR artifacts (side lobes) were filtered as described elsewhere.[18] Only m/z features of monoisotopic candidates were retained in the matrix. Assignment of molecular formulas by mass difference network analysis was performed as recently described.[19]

For van Krevelen diagrams, the intensities of the mass signals measured in either formula-fed (n = 19) or breastfed (n = 16) infants were averaged. Moreover, for better visualization molecular formulas of fatty acids with very high signal intensities were excluded.

UHPLC-MS/MS Analysis. Fecal sample extracts and standard substances (in ACN/H_2O 75:25, v/v) were analyzed by UHPLC (Acquity, Waters, Milford, MA, USA) coupled to a time-of-flight (TOF) mass spectrometer (MS) (maXis, Bruker Daltonics, Bremen, Germany). Hydrophilic interaction liquid chromatography (HILIC) was performed using an iHILIC-Fusion UHPLC column SS (100 × 2.1 mm, 1.8 μm, 100 Å, HILICON AB, Umea, Sweden). Chromatographic settings were the same as previously described[20] with some modifications (injection volume of 5 μL; eluent A consisted of 5 mmol/L NH_4Ac (pH 4.6) in 95% ACN (pH 4.6) and eluent B consisted of 25 mmol/L NH_4Ac (pH 4.6) in 30% ACN with a runtime of 12.1 min, followed by reconditioning for 5 min after each sample, respectively). Every tenth injection a pooled fecal sample was used as a quality control for subsequent batch normalization.

Calibration of the MS was done by injecting ESI-L Low Concentration Tuning Mix (Agilent, Santa Clara, CA, USA) prior to the measurements. Additionally, ESI-L Low Concentration Tuning Mix (diluted 1:4 (v/v) with 75% ACN) was injected in the first 0.3 min of each UHPLC-MS run by a switching valve for internal recalibration. Mass spectra were acquired in positive and negative electrospray ionization mode (±ESI). Parameters of the ESI source were a nitrogen flow rate of 10 L/min, a dry heater set at 200 °C, a nebulizer pressure of 2 bar, and a capillary voltage of 4000 V. Data were acquired in line and in profile mode with an acquisition rate of 5 Hz within a mass range of 50−1500 Da. Data-dependent MS/MS experiments were performed in automated MS/MS mode. After each precursor scan, the five most abundant ions (absolute intensity threshold ≥ 2000 au) were subjected to MS/MS. Each fecal sample was measured in duplicates with a collision energy of 10 and 35 eV, respectively.

Raw UHPLC-MS data were processed with Genedata Expressionist Refiner MS 11.0 (Genedata GmbH, Munich, Germany), including chemical noise subtraction, intensity cutoff filter, calibration, chromatographic peak picking, deisotoping, and metabolite library search against the Human Metabolome Database (HMDB)[21] for MS1 level spectra (±0.005 Da). Peak areas of duplicates were averaged and normalized to fecal weight. Batch normalization based on consecutive quality control measurement samples (pooled sample) was performed after missing value imputation (randomized number between 1.0 and 1.2, based on lowest value of the data matrix).

Targeted MS/MS experiments of different Amadori products were performed in multiple reaction monitoring mode (MRM) at 20 eV in positive ionization mode. The MS/MS fit score between standards and Amadori products in fecal samples were calculated with the Bruker Compass DataAnalysis 4.2 software (Bruker Daltonics, Bremen, Germany). The score indicates how well the masses and intensities of the reference compound spectra agree with those found in the spectrum of a pooled fecal sample.

Preparation of Reference Amadori Products. Glucose was mixed with either leucylisoleucine or lysine, and lactose was mixed with either leucylisoleucine or lysine (1 mL, 0.2 mol/L in Milli-Q purified water, respectively). The four reaction mixtures were heated in closed glass vials for 1 h at 100 °C,[22] cooled to room temperature, and stored at −20 °C. For quantitative measurements *N*-deoxyfructosylleucylisoleucine (FruLeuIle) was purified by diluting the mixture 1:5 (v/v) with Milli-Q water and subsequent isolation by HPLC-MS. The isolated fractions containing FruLeuIle were pooled and dried using a vacuum Schlenk line. The dried product was dissolved in Milli-Q water. Finally, the concentration of the FruLeuIle stock solution was determined with NMR (0.73 mmol/L).

Isolation of *N*-Deoxyfructosyl-/*N*-Deoxylactulosylleucylisoleucine from Feces. To analyze the structures of *N*-deoxyfructosyl- and *N*-deoxylactulosylleucylisoleucine (FruLeuIle and LacLeuIle) by NMR spectroscopy, 6 fecal samples (methanol extracts) from formula-fed infants were selected. The Amadori products were isolated from in total 1 mL (50 mg feces/mL methanol) of the fecal extracts by HPLC-MS. Afterward, the fractions containing either FruLeuIle or LacLeuIle were pooled and evaporated under vacuum at 40 °C (SpeedVac Concentrator, Savant SPD121P, ThermoFischer Scientific, Waltham, MA, USA).

High-Pressure Liquid Chromatography-Based Isolation of Amadori Products. The Amadori products FruLeuIle and LacLeuIle were semi-preparatively purified by high-pressure liquid chromatography (HPLC) using an Agilent 1290 Infinity LC system (Santa Clara, CA, USA) with an Xbridge Prep C8 column (5 μm, 10 × 100 mm, Waters (Milford, MA, USA)). An isocratic method with 93% 5 mmol/L NH_4Ac and 0.1% acetic acid in water and ACN/H_2O 7:93 (v/v) with a runtime of 14 min was used. The flow rate, column temperature, and injection volume was 3 mL/min, 60 °C, and 100 μL, respectively. The sample manager was cooled to 5 °C. The Amadori products were collected with a Gilson 215 Liquid Handler (Middleton, WI, USA), while the specific collection time windows were monitored with an amaZon ETD ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Therefore, the flow was split 10:1 post-column (QuickSplit, ASI, Richmond, CA, USA).

Amadori Product Quantification. Stock solutions of the reference compounds FruLeuIle and FruLys were prepared in Milli-Q water and stored at −20 °C until analysis. The concentration of the stock solution of FruLeuIle was determined as 0.73 mmol/L and of FruLys as 12.4 mmol/L using qNMR. For quantification of FruLeuIle and FruLys in fecal samples, the stock solutions were diluted with ACN/H_2O 75:25 (v/v), and a six-point calibration curve was prepared with a concentration range of 0.073−17.3 μmol/L for FruLeuIle and of 1.24−124 μmol/L for FruLys. Six representative formula-fed children (n = 3 F− and n = 3 F+) were selected randomly, and fecal samples from months 1, 3, 5, 7, 9, and 12 were diluted 1:20 (v/v) with ACN/H_2O

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75:25 (v/v) and analyzed with HILIC UHPLC-TOF-MS (positive ionization mode) as described in the main part. The data were processed with QuantAnalysis 2.2 (Bruker Daltonics GmbH, Bremen, Germany) using a quadratic curve fit.

Nuclear Magnetic Resonance Spectroscopy. The chemical standard leucylisoleucine, the prepared and purified FruLeuIle, and the isolated Amadori products FruLeuIle and LacLeuIle from feces were each dissolved in 70 μL of aqueous NMR buffer (15% D_2O containing TSP) and transferred to 2 mm outer diameter NMR vials. NMR experiments were carried out on a Bruker 800 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 800.35 MHz and equipped with a quadrupole inverse cryogenic probe. The spectrometer used a standard one-dimensional (1D) pulse-sequence noesygppr1d [recycle delay (RD)-90°-t1-90°-mixing time (tm)-90°-acquire] free-induction decay with water presaturation during a relaxation delay of 2 s, a mixing time (tm) set to 200 ms, and a 90° pulse of 9.6 μs. Spectra were acquired with 1024 scans into 64 K data points with a spectral width of 12 ppm. For 2D NMR spectra, phase-sensitive sensitivity-improved 2D TOCSY data with water suppression by gradient-tailored excitation (Watergate) (3-9-19) and using decoupling in the presence of scalar interactions (DIPSI-2) were acquired. For each spectrum, 19228 × 1024 data points were collected, with an acquisition time of 1 s, 16 dummy scans, and a mixing time of 20 ms. The number of scans was adjusted according to the concentration of the sample, ranging from 8 to 256 scans. The spectral widths were set to 12 and 12 ppm in the F2 and F1 dimensions, respectively.

Additionally, quantitative NMR (qNMR) measurements were performed to determine the exact concentrations of the FruLeuIle and FruLys stock solutions. For this, 50 μL of D_2O solvent containing 1 mg/mL internal standard TSP was mixed with 150 μL of the FruLeuIle or FruLys stock solution and transferred to a 3 mm NMR tube. ^{1}H NMR experiment (noesygppr1d) with 128 scans, 16 dummy scans, and a relaxation delay of 30 s was performed, achieving full relaxation of all protons and therefore was a quantitative experiment. All other parameters were set as described previously. Peaks were integrated with TopSpin, and the integrals were calibrated to TSP peaks (9H) and stoichiometrically corrected.

Other Methods. Details of acid hydrolysis of infant formula are given in the Supporting Information.

RESULTS AND DISCUSSION

Nontargeted Metabolomic Screening of Feces from Formula-Fed and Breastfed Infants using FT-ICR-MS. Stool samples of one-month-old children fed either with breastmilk or infant formula were measured by FT-ICR-MS. Assigned molecular formulas were visualized in van Krevelen diagrams to represent a large amount of compounds in a two-dimensional space using the H/C and O/C ratios of molecular formulas.[23] Unique signatures were found for formula-fed (Figure 1) and breastfed (Figure S1) infants with dominant distinctions in nitrogen containing compounds (CHON; orange). In formula-fed infants the O/C ratios between 0.4 and 0.7 were very prominent. The most intense CHON compounds were assigned to $C_{18}H_{34}N_2O_8$, $C_{24}H_{44}N_2O_{13}$, $C_{12}H_{24}N_2O_7$, and $C_{18}H_{34}N_2O_{12}$ (Figure 1). Mass-signal-based annotation using the Human Metabolome Database (HMDB) resulted in only one match, namely for fructosyllysine (FruLys, $C_{12}H_{24}N_2O_7$), which is a well-known Amadori product in human nutrition.[24] Therefore, we suggested that the other abundant CHON containing metabolites could also belong to the class of Amadori products. Amadori products are formed during heating or storage processes of food. In the initial stage of the Maillard reaction, the free ε-amino group of lysine in food proteins reacts with reducing sugars, such as glucose or lactose, resulting in the formation of, for example, FruLys.[24] The exclusively formula-fed infants from this cohort consumed infant formula, which was supplemented with whey powder.

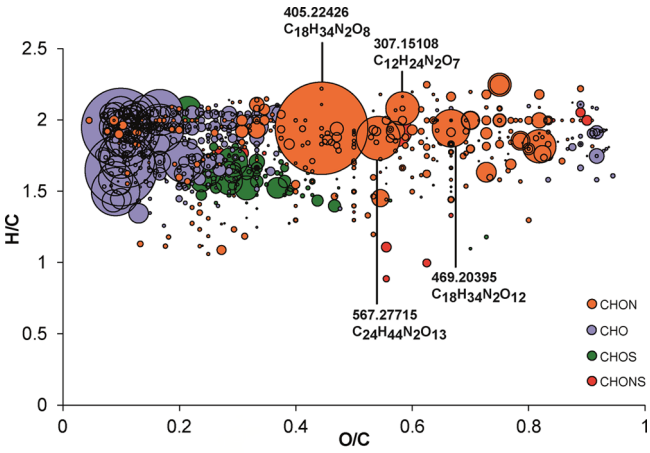

Figure 1. Chemical composition of feces from 1-month-old infants, who were exclusively fed with infant formula (average of signal intensity of $n = 19$ fecal samples), acquired by FT-ICR-MS in negative ionization mode. Calculated molecular formulas were categorized into four compositional groups: CHO (blue), CHON (orange), CHOS (green), and CHONS (red). The van Krevelen diagram plots the H/C vs O/C atomic ratios of the computed molecular formulas. Putative Amadori products are highlighted with measured m/z values and calculated molecular formulas.

Supplementation of whey is frequently used to adapt the cow's milk casein to whey ratio (82:18) to that of human milk (40:60).[25] Whey proteins are richer in lysine than casein, and it can be expected that more Amadori products are formed.[26] Up to 20% of the lysine residues in cow milk-based formula are blocked by Amadori products.[27] The main Amadori product in infant formula is LacLys, which results from the condensation of lactose with lysine residues (lactosylation) of milk proteins, especially with those of the major whey protein β-lactoglobulin. The LacLys content is approximately 3-fold higher in infant formula than in UHT-sterilized liquid cow milk, since infant formulas are more rigorously heat treated for microbiological safety and long shelf life.[28] Heat-induced lactosylation of β-lactoglobulin was found at several lysine residues and the N-terminal leucine.[29−34]

Our assumption of possible Amadori products in stool samples of formula-fed infants was supported by further UHPLC-MS/MS measurements. We analyzed all stool samples and screened them for mass signals, representing the four most intense CHON-containing compounds found by FT-ICR-MS analysis (Figure 1). Due to the polar characteristics of Amadori products, we used a HILIC UHPLC-MS/MS method. An exemplary chromatogram of a fecal sample from one formula-fed infant at the age of one month is shown in Figure 2. All four mass signals were detected within an error of ±0.005 Da ($C_{18}H_{34}N_2O_8$, pink; $C_{24}H_{44}N_2O_{13}$, blue; $C_{12}H_{24}N_2O_7$, green; $C_{18}H_{34}N_2O_{12}$, orange). A typical fragment observed for $C_{18}H_{34}N_2O_8$ and $C_{24}H_{44}N_2O_{13}$ with m/z 245.1860 in MS/MS experiments (Figure 3C and G) was annotated as leucylisoleucine, which coincides with the N-terminal amino acid sequence of β-lactoglobulin. Also, the calculated molecular formulas from the FT-ICR-MS screening supported the assumption of leucylisoleucine Amadori products with glucose and lactose ($C_{18}H_{34}N_2O_8$ and $C_{24}H_{44}N_2O_{13}$). Interestingly, only the dipeptide leucylisoleucine Amadori products and no mass signals for fructosyl-/ lactulosylleucine were detected in feces. To verify our assumptions reference substances were prepared and isolated.

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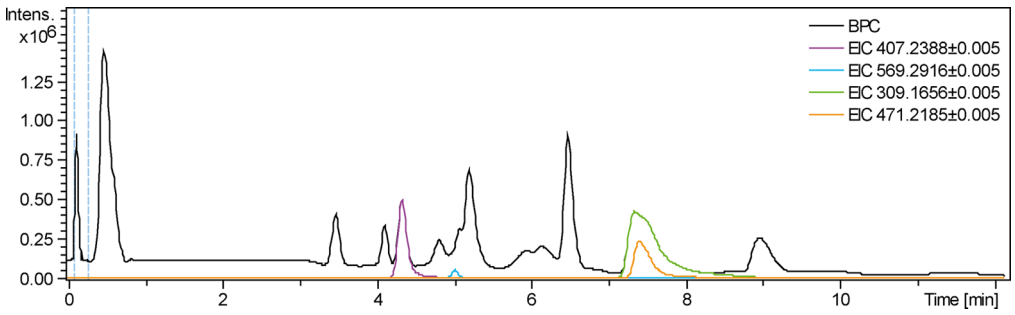

Figure 2. Representative chromatogram of a fecal sample (child ID 25, age 1 month, formula-fed) separated by hydrophilic interaction liquid chromatography (HILIC) and detected in positive ionization mode by TOF-MS. Base peak chromatogram (BPC, black) and extracted ion chromatograms (EICs) of the m/z values referring to the putative Amadori products FruLeuIle ($[M + H]^+$ = 407.2388, pink), LacLeuIle ($[M + H]^+$ = 569.2916, blue), FruLys ($[M + H]^+$ = 309.1656, green), and LacLys ($[M + H]^+$ = 471.2185, orange); extracted with ±0.005 Da.

Identification of Fecal Amadori Products using UHPLC-MS/MS and NMR. Chemical structures were confirmed by comparison of retention time, MS/MS fragmentation patterns, and spiking experiments. The prepared reference compounds FruLeuIle and LacLeuIle (Figure 3A and E, blue) showed the same retention time as the according peak in the fecal sample (Figure 3A and E, orange), which was further confirmed by spiking of purified reference compounds into fecal samples (Figure 3A and E, green). Additionally, the MS/MS fragmentation patterns were the same (fit score of 99.7% and 99.3%, respectively) (Figure 3B, C and F, G). Chemical structures of FruLeuIle and LacLeuIle are shown in Figure 3D and H. Matching retention times and MS/MS fragments between the reference substances of FruLys (purchased) and LacLys (prepared reference) and the pooled fecal sample from formula-fed infants are illustrated in Figure S2. All Amadori products showed similar fragmentation patterns (Figure 3B, D and Figure S2B, D). Dominant fragment ions were neutral losses of water [(18 Da ($-H_2O$), 36 Da ($-2H_2O$), 54 Da ($-3H_2O$)); 84 Da ($-3H_2O-CH_2O$)] and loss of the glucose/lactose moiety (162/324 Da), which are characteristic for this compound class.[3,35,36]

Furthermore, FruLeuIle and LacLeuIle were isolated from feces by HPLC, and the structures were verified by NMR spectroscopy (1D ^{1}H Figure 4 and 2D ^{1}H−^{1}H TOCSY Figures S3−S6). First, NMR spectra of leucylisoleucine were recorded to annotate chemical shifts of each hydrogen atom (Figures 4A and S3). These were 0.893 (3H, t, H-1), 0.925 (3H, d, H-4), 0.967 (6H, dd, H-9, H-10), 1.175 (1H, m, H-2), 1.463 (1H, m, H-2), 1.711 (2H, m, H-7, H-8), 1.788 (1H, m, H-7), 1.832 (1H, m, H-3), 4.069 (1H, t, H-6), and 4.116 (1H, dd, H-5). Second, the prepared product of leucylisoleucine and glucose, i.e., FruLeuIle was analyzed and compared to the unconjugated dipeptide (Figures 4B and S4). Slight variations were observed for the chemical shifts, introduced by the conjugation of glucose near H-6 but separated by NH. These were 0.893 (3H, t, H-1), 0.925 (3H, d, H-4), 0.967 (3H, d, H-9 or H-10) and 0.933 (3H, d, H-9 or H-10), 1.19 (1H, m, H-2), 1.463 (1H, m, H-2), 1.665 (1H, m, H-8), 1.688 (1H, m, H-7), 1.833 (1H, m, H-7), 1.85 (1H, m, H-3), 4.012 (1H, t, H-6), and 4.164 (1H, dd, H-5). In addition, a doublet of doublets was observed, arising from the CH_2 connecting the dipeptide and monosaccharide (3.19 (2H, dd, H-11)). Further signals arising from the bound glucose were observed, i.e., 3.70 (1H, m, H-14), 3.73 (1H, dd, H-12), 3.88 (1H, dd, H-13), and 4.01 (2H, m, H-15). Subsequently, these shifts were compared to resonances arising from the proposed FruLeuIle in fecal extracts (Figures 4C and S5). Here again similar resonances were found, confirming the presence of the Amadori product in stool, i.e., 0.893 (3H, t, H-1), 0.925 (3H, d, H-4), 0.967 (3H, d, H-9 or H-10) and 0.933 (3H, d, H-9 or H-10), 1.19 (1H, m, H-2), 1.463 (1H, m, H-2), 1.66 (1H, m, H-8), 1.719 (1H, m, H-7), 1.788 (1H, m, H-7), 1.85 (1H, m, H-3), 4.07 (1H, t, H-6), and 4.164 (1H, dd, H-5). For LacLeuIle, similar chemical shifts were found, with an additional resonance at 4.52 ppm (1H, d, H-16) from the galactose moiety (Figure S6).

To verify the presence of Amadori products in the infant formula consumed in this cohort, the formula powder was acid hydrolyzed. Under these conditions Amadori products are released from milk proteins in the form of *N*-(2-furoylmethyl) amino acids.[2,28,37] Accordingly, mass signals for *N*-(2-furoylmethyl)-lysine (furosine), *N*-(2-furoylmethyl)-leucine, and *N*-(2-furoylmethyl)-leucylisoleucine were detected. Their fragmentation patterns showed typical fragments of lysine (84.082 Da) and leucine/isoleucine (86.097 Da) (Figure S7), which were also present in the Amadori product fragmentation patterns. Penndorf et al.[37] identified several *N*-(2-furoylmethyl) amino acids in hypoallergenic but only furosine in conventional (intact protein-containing) infant formulas. However, the detection of *N*-(2-furoylmethyl)-leucine and *N*-(2-furoylmethyl)-leucylisoleucine in our study might be due to different detection methods and general variations between different infant formulas (e.g., differences in whey supplementation).

Fecal Excretion Profiles of the Change of Amadori Products over Time. The fecal excretion of the identified Amadori products in relation to formula consumption was studied in infants within the first year of life (six time points) and at the age of 24 months (no longer formula-fed) by HILIC UHPLC-MS (Figure 5, Table S1). Exclusively breastfed infants served as the control group (B, red). In formula-fed infants (F−, green and F+, blue) the Amadori products were most abundant at the age of 1−3 months. Apart from FruLeuIle, we noticed a strong decrease in Amadori profiles after an age of 5 months. By comparison, FruLeuIle showed relatively high values up to an age of 9 months (Figure 5A). Most of the formula-fed infants received additionally solid food from month 5 onward (Table S1), which most likely contributed to the reduced excretion of the Amadori products. The lactosylated compounds (Figure 5B and D) declined earlier than the glycated ones (Figure 5A and C), which might indicate an enhanced capacity for the digestion of the lactose moiety progressing with time. At the age of 12 months, toddlers receive a much lower amount of formula in relation to their body weight compared to newborns and are fed primarily with solid food. Therefore, most Amadori products

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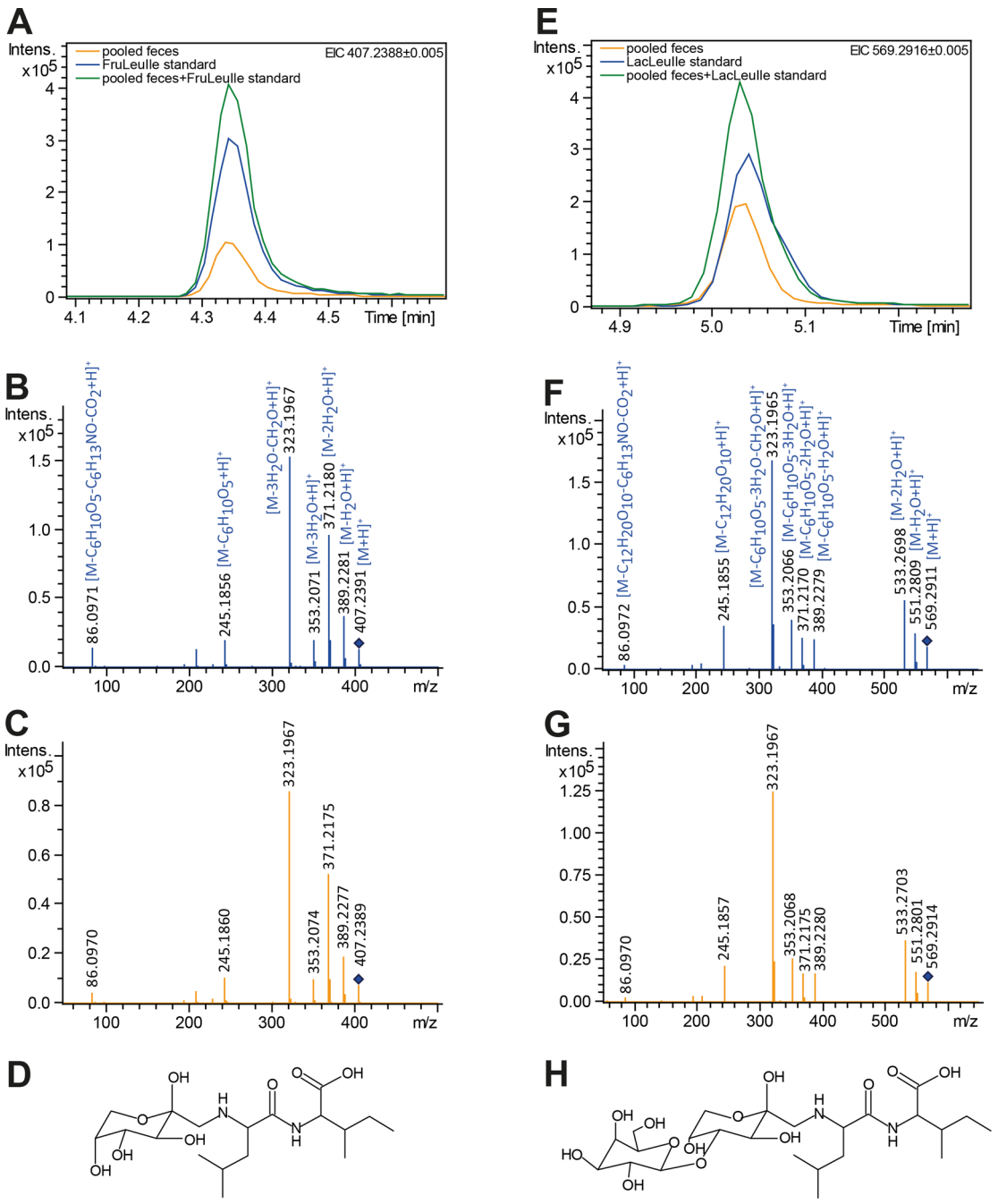

Figure 3. Identification of LeuIle Amadori products performed with HILIC LC-MS/MS (positive ionization mode). (A) Extracted ion chromatogram (EIC) of FruLeuIle ($[M + H]^+$ = 407.2388 ± 0.005 Da) in feces (orange), prepared reference compound (blue), and spiking of the purified Amadori product into the pooled fecal extract (green). (B) Collision-induced dissociation MS/MS experiments (20 eV) of the prepared FruLeuIle standard and (C) of pooled fecal samples (MS/MS match: fit score of 99.7%). (D) Proposed chemical structure of FruLeuIle. (E) EIC of LacLeuIle ($[M + H]^+$ = 569.2916 ± 0.005 Da) in feces (orange), prepared reference compound (blue), and spiking of the purified Amadori product into the pooled fecal extract (green). (F) Collision-induced dissociation MS/MS experiments (20 eV) of the prepared LacLeuIle standard and (G) of pooled fecal samples (MS/MS match: fit score of 99.3%). (H) Proposed chemical structure of LacLeuIle.

were not detectable any more. Interestingly, FruLeuIle was still detectable in low amounts at an age of 12 months (Figure 5A). Besides the displacement of the formula-derived Amadori products by feeding of solid food, the gut microbiota could also play an important role. During the first year of life the gut microbiome significantly changes and becomes more diverse.[16] Many studies have suggested a microbial degradation of FruLys in the intestinal tract[6−10,38−41] and modulation of the microbiota composition by Maillard reaction products.[42] This hypothesis was proven for specific bacterial strains[43−45] and for fecal suspensions from human adults.[46] Whether formula-derived Amadori products have an influence on the composition of the infant's gut microbiota or not needs to be investigated. At the age of 24 months Amadori products were no longer detectable. Overall, there were no major differences between the formula-fed group with probiotics (F+, blue, Figure 5) and without probiotics (F−, green; Figure 5). However, the high variation between the different samples of the same month

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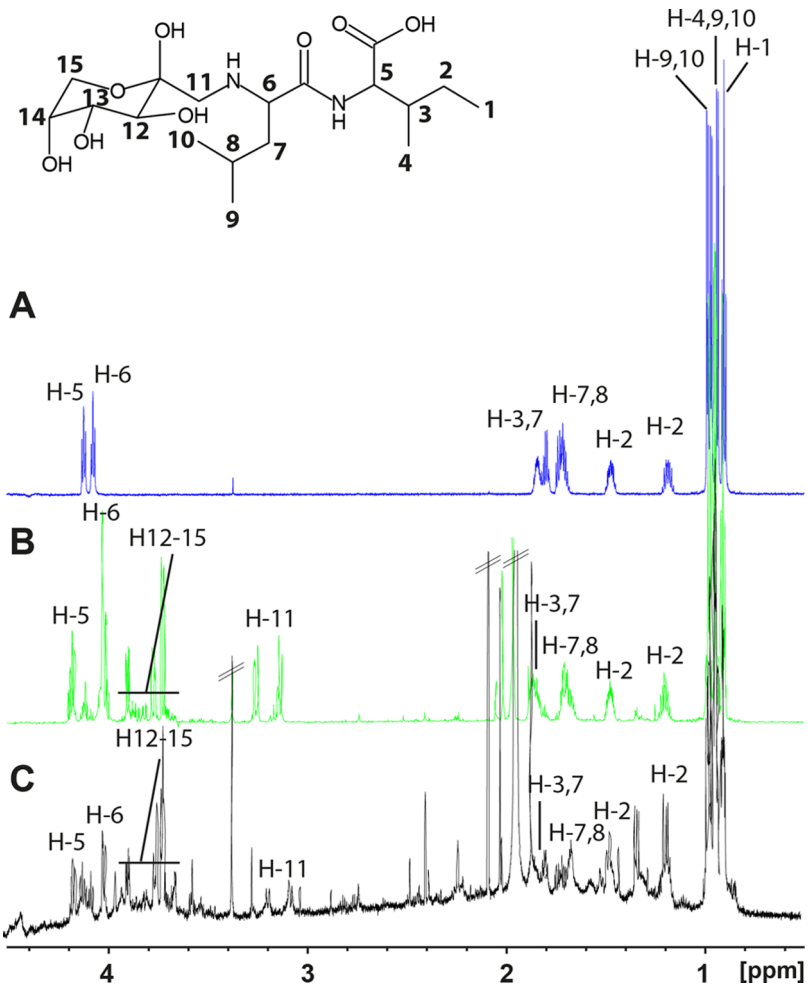

Figure 4. Identification of FruLeuIle Amadori products performed with ^{1}H NMR spectroscopy and structural annotation. (A) LeuIle standard, (B) prepared FruLeuIle standard, and (C) extracted FruLeuIle from fecal samples. Small deviations in the chemical shift for FruLeuIle derive from different amounts of NH_4Ac in the two samples.

indicate large interindividual differences. During exclusive breastfeeding no Amadori products were excreted in feces (B, red; Figure 5). However, after the introduction of solid food, we could detect low amounts of Amadori products occasionally, which may originate from other milk sources.

To get a better idea of the quantities of excreted Amadori products in feces, samples from 6 formula-fed individuals were selected and FruLeuIle and FruLys were quantified in samples from month 1 to 12 (Figure S8). The concentrations of the stock solutions used for quantification were determined with qNMR (Figure S9). In total, the fecal concentrations of the two Amadori products were high during the first months of life and much lower in months 9 and 12. On average 1.6 μmol/g of FruLeuIle (Figure S8A) and 15.7 μmol/g of FruLys (Figure S8B) were excreted into feces at the age of 3 months. Overall, the concentration of FruLys was much higher than the concentration of FruLeuIle. This can be explained due to the fact that the consumed amount of protein-bound FruLys was higher due to multiple lysine residues in whey and casein proteins compared to only one N-terminal glycation site in β-lactoglobulin resulting in FruLeuIle. Nevertheless, the excretion of the two Amadori products show interindividual differences; for example, for Child 27 and 75 the FruLys/FruLeuIle ratio is lower than for the others. This could be due to individual absorption and excretion kinetics. In rats the fecal excretion of FruLys and LacLys was reported to be much lower than in urine.[7,10,47,48] Yet, about 95% of the ingested FruLys was neither found in urine nor in feces and might be metabolized in yet unknown pathways.[10,49] However, in human infants this ratio seems to be different. Niederweiser et al.[50] determined a fecal excretion rate of 55% and only 16% in urine for FruLys in infants. Formula-fed preterm infants excreted 1.3−3.9% of the ingested amount of LacLys via urine.[51] The reason for the higher concentrations of FruLys in stool of infants might originate from different microbiota composition compared to adults or rodents. The early life microbiota might not be adapted to the intake of heat-treated proteins, and therefore, degradation is less efficient.[47] Another reason might be that infants absorb compounds with higher molecular weights more efficiently.[10] However, no data is available for the absorption and distribution rates of FruLeuIle. Nevertheless, the metabolic transit of Amadori products depends on their chemical structure.

In summary, we identified milk-derived Amadori products in feces of formula-fed infants by combining nontargeted metabolomics and targeted structure elucidation using MS/MS and NMR in a nutritional intervention study. The most probable dietary source of FruLeuIle and LacLeuIle is the heat-treated whey protein β-lactoglobulin in infant formula because of its N-terminal sequence. In general, the augmented fecal excretion of FruLys and FruLeuIle demonstrates the high load of

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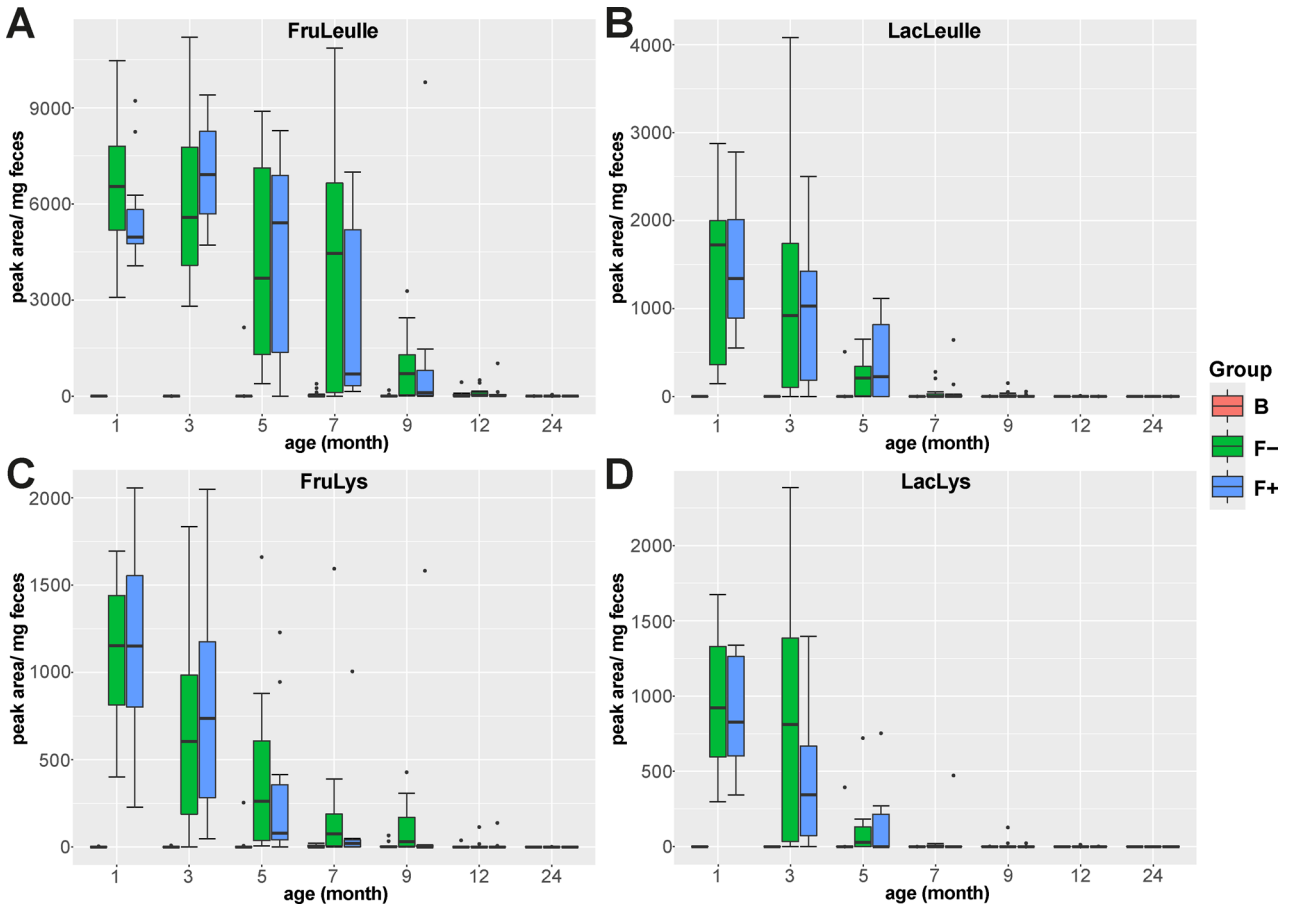

Figure 5. Fecal excretion profiles of the Amadori products (A) FruLeuIle, (B) LacLeuIle, (C) FruLys, and (D) LacLys during the first 2 years of life. In formula-fed infants (F−, without probiotics, green, and F+, with probiotics, blue) the amount of excreted Amadori products decreased over time. Breastfed infants (B, red) excreted only very small amounts after introduction of solid food (approximately 5 months).

Maillard reaction products to which formula-fed infants are exposed during the first months of life. The absorption of the different Amadori products in the gastrointestinal tract and potential modulation of the infants gut microbiota remains to be investigated in future studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b01889.

Methodical details of acid hydrolysis of infant formula; van Krevelen diagram of feces from breastfed infants (Figure S1); LC-MS/MS identification of FruLys and LacLys (Figure S2); 2D-^{1}H−^{1}H-TOCSY NMR spectra of leucylisoleucine (Figure S3), prepared reference compound FruLeuIle (Figure S4), FruLeuIle (Figure S5), and LacLeuIle (Figure S6) isolated from feces; fragmentation patterns of *N*-(2-furoylmethyl) amino acids in infant formula after acid hydrolysis (Figure S7); quantitative excretion profiles of FruLeuIle and FruLys in selected samples (Figure S8); and qNMR measurements of FruLeuIle and FruLys stock solutions (Figure S9) (PDF)

Information on infant cohort and HILIC UHPLC-QTOF-MS analysis of Amadori products in fecal samples (Table S1) (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: alesia.walker@helmholtz-muenchen.de. Tel.: +49 89 3187 4656.

ORCID

Alesia Walker: 0000-0002-1477-2023

Present Address

⊥Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit and Umwelt, Department of Environmental Sciences, Research Unit Analytical BioGeoChemistry, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ACN, acetonitrile; B, exclusively breastfed; ESI, electrospray ionization; F−, formula-fed without probiotics; F+, formula-fed with probiotics; FruLeuIle, *N*-deoxyfructosylleucylisoleucine; FruLys, *ε*-*N*-deoxyfructosyllysine; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; LacLeuIle, *N*-deoxylactulosylleucylisoleucine; LacLys, *ε*-*N*-deoxylactulosyllysine; MS, mass spectrometry/spectrometer; MS/MS, tandem mass spectrometry; *m*/*z*, mass-to-charge ratio; NH_4Ac, ammonium acetate; (q)NMR, (quantitative) nuclear magnetic resonance spectroscopy; TSP, trimethylsilylpropionate; UHPLC, ultra-

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high-performance liquid chromatography; UHT, ultrahigh temperature

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