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Intestinimonas-like bacteria are important butyrate producers that utilize *N*ε-fructosyllysine and lysine in formula-fed infants and adults

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ABSTRACT

Our study aim is to investigate the role of *Intestinimonas, N*ε-fructosyllysine (FL)-degrading bacterium, in infants and adults. We used lysine and subsequently FL in anaerobic serial dilutions of stools of infants and adults to enrich lysine and FL-degrading species. The fecal microbiota of adults were able to ferment lysine and FL to butyrate. Different groups of *Intestinimonas* spp. were detected from all lysine enrichments whereas the FL enrichments consisted of broader taxonomic groups with a reduced abundance of *Intestinimonas*-related species. Remarkably, the capability to degrade FL was only observed in formula-fed but not in breast-fed infants, which may relate to high contents of FL in formulas after thermal treatment. This possibility was supported by analyzing metagenomic datasets of 3-month and 4-month infants. Our data indicate the key role of *Intestinimonas*-like bacteria in FL degradation in formula-fed infants and adults as a profound example of adaptation of intestinal bacteria to dietary components.

1. Introduction

The thermal treatment of foods containing reducing carbohydrates and protein results in the formation of dietary advanced glycation end-products (d-AGEs) (Delgado-Andrade & Fogliano, 2018). Most of these d-AGEs are poorly bioavailable and cannot be used for *de novo* protein synthesis. The nutritional and health concerns regarding the food thermal treatment are related to the decrease of available lysine, especially in infant formulas, and to the metabolic fate of the d-AGEs (Erbersdobler & Somoza, 2007, Pischetsrieder & Henle, 2012). Amadori products are the first stable compounds formed through the Maillard reaction upon the rearrangement of the Schiff base between reducing carbonyls and amino group (Yaylayan, Huyghues-Despointes, & Feather, 1994). The Amadori products are by far the most abundant glycation compounds present in foods, especially *N*ε-fructosyllysine (FL). Heat treatments during milk processing has a great impact on the nutritional quality of the milk, resulting in a large amount of Amadori products in the processed milk including infant milk formulas (Hegele, Buetler, & Delatour, 2008, Pischetsrieder & Henle, 2012).

Protein glycation through Maillard reaction greatly enhances the amount of proteins reaching the gut microbiota since lysine blockage hinders one of the peptide bonds most commonly targeted by digestive proteases (Snelson and Coughlan, 2019). Hence, glycation promotes protein crosslinking, decreasing overall digestibility and funneling additional material to the colon. The amount of protein bound in d-AGEs is inversely correlated with protein digestibility and it has been estimated that a daily intake of around 0.5–1 g of Amadori products is available as nitrogen source by the microbial populations (Tuohy et al., 2006). Both intact proteins and glycated proteins can be metabolized by intestinal bacteria (Portune et al., 2016) and increasing evidence suggests that a diet rich in post-translational modified proteins profoundly changes the microbiota composition and intestinal bacteria are able to

Abbreviations: d-AGEs, dietary advanced glycation end-products; FL, *N*ε-fructosyllysine, LC-MS/MS, liquid chromatography tandem mass spectrometry; HRMS, high resolution mass spectrometry

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use some of glycated compounds as substrate (Delgado-Andrade, Tessier, Niquet-Leridon, Seiquer, & Pilar Navarro, 2012, Hellwig et al., 2015).

In a recent study FL was given to gnotobiotic mice, colonized with 54 phylogenetically diverse human gut bacterial strains, resulting in an increased abundance of *Collinsella intestinalis* combined with the induction of the expression of genomic loci involved in FL metabolism (Wolf, Wesener, & Cheng, 2019). However, no butyrate was detected as end product but acetate and formate. This pathway has been well described in *Escherichia coli* and it has been recently demonstrated that FL metabolism is tightly controlled at the transcriptional level by global and substrate-specific regulators in *E. coli* (Wiame, Delpierre, Collard, & Van Schaftingen, 2002; von Armansperg, Koller, Gericke, Hellwig, Jagtap, Heermann, Hennig, Henle, and Lassak, 2020). However, the used consortium did not include *E. coli* nor butyrogens that can utilize FL, as we recently described (Bui et al., 2015). Recently, we showed that intestinal bacteria are also able to degrade *N*ε-carboxymethyllysine (CML) which is one of the most stable d-AGEs in anaerobic conditions (Bui, Troise, Fogliano, & de Vos, 2019). We found uncultured *Oscillibacter* spp. that was enriched in CML from one human volunteer and isolated *Cloacibacillus evryensis* strain that converted CML to carboxymethylated biogenic amines and carboxylic acids. In another study, an increased production of butyrate has been reported in rat that were fed with glycated fish proteins (Han et al., 2017, Han et al., 2018). These findings can be well explained by the activity of *Intestinimonas butyriciproducens*, a human intestinal isolate capable of the complete anaerobic conversion of 1 mol of FL into 3 mol butyrate, in a unique pathway that we described previously in molecular detail (Bui et al., 2015). It was observed that all *I. butyriciproducens* strains have the capacity to convert FL as well as lysine into butyrate using this pathway (Bui et al., 2016). The presence in the human intestinal tract of bacteria with the same physiology as *I. butyriciproducens* could result in the efficient use of FL, a hypothesis which is coherent with the large amount of Amadori products provided through the Western diet. While proteolytic bacteria mostly produce branched chain fatty acids as end products, *I. butyriciproducens* ferments glucose and several other sugars to butyrate. The presence of trypsin in the gut that cleaves protein and peptides at lysine residues would also increase the amount of modified free lysine in the colon that are available for conversion by *I. butyriciproducens* (Bui et al., 2015).

In this work, a strategy of applying successive transfers of intestinal bacteria from human stools in anaerobic growth media enriched with lysine and subsequently FL was adopted to identify species able to metabolize FL. Beside the isolation and characterization of these bacterial species, we aimed at ascertaining if the ability of degrading FL by *Intestinimonas* spp. is related to the dietary exposure in young infants before weaning.

2. Material and methods

2.1. Chemicals and reagents

Acetonitrile, methanol and water for mass spectrometry analyses were obtained from Merck (Darmstadt, Germany). Ammonium hydroxide, ammonium formate, D-glucose, Fmoc- α-protected L-lysine and d4-lysine were purchased from Sigma-Aldrich (St. Louis, MO). Nε-fructosyllysine (FL) was purchased from TRC (North York, ON).

2.2. Synthesis and purity of Nε-fructosyllysine (FL)

In order to obtain sufficient amount of substrate, FL was synthesized as previously reported (Troise, Wiltafsky, & Fogliano, 2015) starting from Fmoc-α-protected L-lysine that reacted specifically on its epsilon amino moiety with D-glucose in refluxed methanol and subsequently de-protected under basic conditions to afford the desired product. The identity, isotopic distribution and the elemental analysis ($C_{12}H_{24}N_2O_7$) of the obtained FL was checked by direct infusion (3 μL/min) onto high resolution mass spectrometer (Exactive, Thermo Fisher, Bremen, Germany). A purity of ≥95% ($[M+H]^+$ 309.16551, mass error −0.4 ppm) was found in agreement with previously reported data of Amadori compounds and with pure FL reference standard.

2.3. Enrichment and growth media

The study involved 6 subjects (F1, M1, M2, AF1A, AF4A, AS1) with inform consents as reported previously (Bui et al., 2019). Fresh fecal samples were collected in 15 mL conical tubes containing anaerobic phosphate buffer (pH 7) and later stored in 25% glycerol in 5 mL anaerobic bottles kept at −80 °C freezer; 0.5 mL of these fecal slurries was transferred to 10 mL anaerobic bicarbonate-buffered mineral salt medium (CP medium) containing 40 mM lysine as energy and carbon source to enrich lysine-fermenting bacteria. The head space was filled with CO_2/N_2 (1:4) at 1.5 atm and incubation was at 37 °C. For positive growth dilutions, the cultures were transferred to new media to eliminate the influence of fecal materials from the fresh samples and glycerol content from frozen stocks on the lysine fermentation capability. Subsequently, the enrichment cultures were transferred two more times in the same lysine-containing medium before being transferred to FL. Due to the limitation on quantity of FL, this substrate was dissolved in CP medium and filter sterilized in anaerobic serum bottles while L-lysine was added into the bicarbonate buffered medium from 1 M sterile anoxic stock solution. The enrichment of lysine-fermenting microorganisms was done in a serial dilution from 10^{-1} up to 10^{-5}. Those cultures were later transferred to CP medium containing 5 mM FL to check for the capacity to convert FL into butyrate.

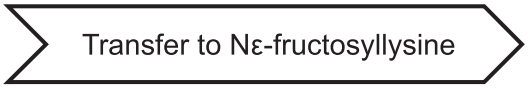
Enrichments were performed in anaerobic bicarbonate-buffered medium consisting of (mg/mL): 0.53 Na_2HPO_4-$2H_2O$, 0.41 KH_2PO_4, 0.3 NH_4Cl, 0.11 $CaCl_2$–$2H_2O$, 0.10 $MgCl_2$-$6H_2O$, 0.3 NaCl, 4.0 $NaHCO_3$ and 0.48 Na_2S-$9H_2O$ as well as alkaline and acid trace elements (each 0.1% v/v) and vitamins (0.2% v/v) (Stams, Van Dijk, Dijkema, & Plugge, 1993). The alkaline trace element solution contained the following (mM): 0.1 Na_2SeO_3, 0.1 Na_2WO_4, 0.1 Na_2MoO_4 and 10 NaOH. The acid trace element solution was composed of the following (mM): 7.5 $FeCl_2$, 1 H_3BO_4, 0.5 $ZnCl_2$, 0.1 $CuCl_2$, 0.5 $MnCl_2$, 0.5 $CoCl_2$, 0.1 $NiCl_2$ and 50 HCl. The vitamin solution had the following composition (g/L): 0.02 biotin, 0.2 niacin, 0.5 pyridoxine, 0.1 riboflavin, 0.2 thiamine, 0.1 cyanocobalamin, 0.1p-aminobenzoic acid, and 0.1pantothenic acid.

2.4. Detection of short chain fatty acids

The formation of short chain fatty acids and alcohols were measured on a Thermo Scientific Spectra HPLC system equipped with a Metacarb 67H 300 × 6.5-mm column (Agilent, Santa Clara, CA) kept at 37 °C and running with sulfuric acid as an eluent. The eluent flow was 0.8 mL/min. The detector was a refractive index detector (RI 150, Thermo Fisher). To obtain a good separation of metabolites, the measurement was performed at 45 °C.

2.5. FL and lysine LC-MS/MS detection

Lysine and FL were analyzed as previously described (Troise, Wiltafsky, Fogliano, & Vitaglione, 2018) with specific optimization of FL detection through direct infusion into a tandem mass spectrometer equipped with a HESI source (TSQ, Thermo Fisher). Samples were diluted five hundred times in acetonitrile:water (50:50, v/v) and hydrophilic interaction liquid chromatography (HILIC) separation of FL, lysine and its labelled internal standard *d4*-lysine was achieved on a thermostated (40 °C) silica Kinetex HILIC (2.6 μm, 75 × 2.1 mm, Phenomenex, Torrance, CA) equipped with a security guard of the same stationary phase. Chromatographic separation of lysine and FL was achieved by an Ultimate 3000 RS (Thermo Fisher Scientific, Bremen,



Sample	Serial dilution				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
F1	+	+	NA	NA	NA
M1	-	-	NA	NA	NA
M2	-	-	-	-	-
AF1A	+	NA	NA	NA	NA
AF4A	+	+	NA	NA	NA
AS1	+	+	+	+	+

Sample	Serial dilution				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
F1	+	+	NA	NA	NA
M1	-	-	NA	NA	NA
M2	-	-	-	-	-
AF1A	+	NA	NA	NA	NA
AF4A	+	+	NA	NA	NA
AS1	+	+	+	+	+

Fig. 1. Lysine and FL enrichment process. Plus (+) indicated the growth while minus (−) indicated no growth observed. The growth was determined by the turbidity of the medium, substrate reduction and metabolite production. The capacity to grow, reduce the substrate ad produce butyrate is indicated in red for lysine and purple boxes for FL growth. NA, not applicable.

Germany) through three mobile phases consisting of 0.1% formic acid in acetonitrile (solvent A), 0.1% formic acid in water (solvent B) and 50 mM ammonium formate (solvent C). The following linear gradient of solvent B (min/%B): (0/2), (1.20/0), (4/40), (6/40) was used, while mobile phase C was constant at 10%. The flow rate was set to 400 µL/min and the injection volume was 5 µL. Positive electrospray ionization was used for detection and the source parameters were selected as follows: spray voltage of 3.5 kV; capillary temperature of 300 °C, vaporizer temperature of 250 °C while auxiliary and sheath gas were set at 60 and 10 arbitrary units, respectively. The chromatographic profile was recorded in selected reaction monitoring mode (SRM). Target compounds were analyzed using the mass transitions given in parentheses along with collision energy (CE) and in bold the transition used for the quantitation in the case of lysine and FL and for qualification in the case of internal standard experiments: lysine (*m/z* 147 → 84, CE: 25 V; **130** CE: 34 V), *d4*-lysine (*m/z* 151 → **88**, CE: 30 V; 134, CE: 16 V), FL (*m/z* 309 → 84, CE: 30 V; **291** CE: 16 V). Analytical performances robustness, sensitivity, reproducibility, repeatability, linearity, accuracy, carry over and matrix effects were evaluated by following the procedures previously reported (Troise et al., 2018). Both lysine and FL were quantified by using the internal standard technique, and the results were reported as mmol/L.

2.6. Bacterial community analysis

Cell biomass from enrichments or pure cultures was harvested from an aliquot (1–2 mL) of well-homogenized liquid culture by centrifuging at 13000*g* for 10 min. DNA was extracted from the pellet by using MasterPure™ Gram Positive DNA Purification Kit (Epicentre, San Diego, CA) as recommended by manufacturer. The PCR was performed using 27F and 1492R (Weisburg, Barns, Pelletier, & Lane, 1991) to amplify the complete 16S rRNA genes of the bacteria using the program starting at 94 °C for 5 min and continued with 35 cycles consisting of 94 °C for 90 s, 52 °C for 30 s and 72 °C for 90 s and finally 72 °C for 10 min. The PCR products were subsequently purified by PCR purification kit (Qiagen, Germany) and used to generate a clone library of full-length 16S rRNA gene sequences using pGEM Easy Vector Systems (Promega, Madison, WI). All steps mentioned above were performed according to manufacturers' instructions. Thirty clones were selected for sanger sequencing at GATC Biotech (Konstanz, Germany) using SP6 (5′-ATTTA GGTGACACTATAGAA-3′) as sequencing primer. The sequences were trimmed with DNASTAR to remove vector contamination and manually checked. Later these sequences were aligned with the multiple sequence aligner SINA (Pruesse, Peplies, & Glöckner, 2012) and merged with the Silva SSU Ref database (release 111). Phylogenetic trees were constructed in the ARB software package (v. 6) by the same algorithm (Ludwig, Strunk, & Westram, 2004).

2.7. Metagenome analysis

The publicly available metagenomic sequence data from two large infant studies (Yassour, Vatanen, & Siljander, 2016, Ferretti, Pasolli, & Tett, 2018) were filtered for stool samples of the 3-month and 4-month infants with their corresponding mothers. Reads were first quality filtered with BBTools function BBduk using the following parameters: ref = adapters.fa ktrim = r k = 23 mink = 11 hdist = 1 tpe tbo. The filtered reads were converted to fasta format and used as an input for DIAMOND blastx against the proteins of interest which was described previously (Bui et al., 2015). Reads having more than 40% identity with a protein were considered as a hit.

3. Results and discussion

3.1. Lysine and FL enrichments using stools of infants and adults

Because of the observed genetic and metabolic association between lysine and FL degradation pathways in butyrate producers (Bui et al., 2015), we first inoculated stool samples of 3 infants and 3 adults in bicarbonate buffer medium containing lysine as sole energy and carbon source from 10^{-1} and 10^{-5} dilution. The growth of each dilution was determined by the turbidity of the cultures, substrate reduction and metabolite production. To eliminate the influence of stool materials on the growth, all grown dilutions were subsequently transferred to fresh media supplemented with lysine and this was the second enrichment (Fig. 1). All samples showed growth at the first lysine enrichment series but two out of six samples (M1 and M2) lost the capabilities of growth in the second enrichment in lysine. As anticipated, all enrichments that grew on lysine, also showed growth on FL when transferred to this substrate (Fig. 1). This indicated the presence of FL-degrading bacteria in the samples F1, AF1A, AF4A and AS1 but not M1 and M2. Of note, samples M1 and M2 derived from infants that had received breast milk. It has been recently shown that a thermal treatment and a type of food were strong selective factors for different groups of intestinal microbes and as a result influenced the microbiota composition and short-chain fatty acid production (Pérez-Burillo et al., 2018). In addition, formula milk has been reported as the major source of the excreted Amadori products in infants (Sillner et al., 2019). The observation that the microbiota of infants only exposed to breast milk were not able to metabolize the FL, while the infant fed with formula milk was, could be a preliminary indication that microbiota can easily adapt to the Amadori

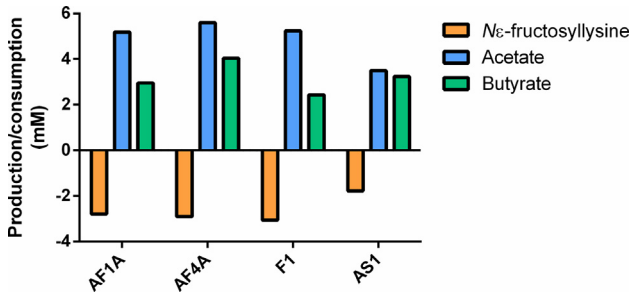


Fig. 2. FL degradation by the highest dilutions of second lysine enrichments from 4 volunteers AF1A, AF4A, F1 and AS1. The measurement was performed after a-week incubation.

products from the diet. During the first years of life, the ability of the human microbiota to use Amadori compounds is significantly related to the type of diets and to the level of glycation of formula milk. The concentration of FL present in the stool at the age of three months was 15.7 μmol/g of feces then it decreased to below detection level after the first year (Sillner et al., 2019). It was suggested the decrease of glycated amino acids in stool samples of these infants was due to the reduced dietary intake of the Amadori products related to weaning. However, an adaptation of microbiota in metabolizing Maillard intermediates as Amadori products was not ruled out. Our data indicate that the reduction of Amadori products in the stool of these formula fed infants after the first year could also well be explained by the adaptation and activity of the microbiota after a long-time exposure to the diets.

All enrichments at the second transfer on lysine produced equal amounts of acetate and butyrate as end products (data not shown), indicating the classical bacterial lysine fermentation by *Intestinimonas* species (Bui, Shetty, & Lagkouvardos, 2016). For those enrichments that the growth on FL was observed, acetate and butyrate were also found as major metabolites after a week incubation (Fig. 2). However, often more acetate than butyrate were formed and this stochiometry differed from the complete conversion into butyrate as reported previously for pure cultures of *Intestinimonas* (Bui et al., 2015). This was somewhat expected as the cultures were not purely *Intestinimonas* species and therefore other strains could have influenced the fermentation profiles. The fact that butyrate was detected as end product indicated that the butyrogenic fermentation pathway from FL was active and suggested the presence of *Intestinimonas* spp. from these enrichments. To confirm this, we performed a DNA-based analysis on the microbial communities of the lysine and FL enrichments.

3.2. Microbial communities in lysine enrichments with Intestinimonas-related species as dominant group

The analysis on the microbial communities of the lysine enrichments for the second transfers was outlined in Fig. 3. As Illumina sequencing only gives short reads which are rather difficult to pinpoint at species level, clone library was chosen to analyze the microbial community. The phylogenetic trees based on 16S rRNA genes were made using 900 bp of the sequences up to 24 clones and related species using ARB (Ludwig et al., 2004). The majority of the bacteria from the lysine enrichments from AF1A, AF4A and AS1 belonged to a group of *Intestinimonas* genus while a several groups of *Intestinimonas*-related species were detected in the enrichment from F1; 13 out of 17 clones were clustered in 3 sub-groups that were 95.6 to 97.9% similar to *I. butyriciproducens* in AS1. All clones in AF4A enrichment were highly similar to *I. butyriciproducens* (97.1–98.8%) and 8 out of 21 clones in AF1A had a similarity from 95.5% till 98% to *I. butyriciproducens*. The microbial community obtained from the enrichment of sample F1 was more diverse and formed 4 separate clades within the *Intestinimonas-Flavonifractor-Pseudoflavonifractor* group. The majority (3 of out 4) of these clades consisted of 16S rRNAs with 94.5–95.7% similarity to that of *I. butyriciproducens*. Recently, it was reported that an isolate carrying a 16S rRNA sequence with a 94.95% similarity to that of *I. butyriciproducens* was found to belong to the *Intestinimonas* genus (Afouda et al., 2019). Hence, it is likely that the clones we observed derive from yet to be characterized *Intestinimonas* spp. This result indicated that bacteria belonging to *Intestinimonas* were involved in the lysine fermentation in the adult intestine while lysine fermentation in infants might spread in a broader taxonomic group of *Intestinimonas* and related genera. To better understand the role of *Intestinimonas* spp. in infants it is necessary to analyze a larger number of volunteers. Nevertheless, the observed butyrogenic activity of infant microbiota is in a good agreement with previous studies that butyrate-producing bacteria appeared at a detectable level after 4 to 12 months after birth (Bäckhed, Roswall, & Peng, 2015, Yassour et al., 2016).

The analysis on the microbial composition was subsequently performed in the 4 enrichments and the results are presented in **Figure S1-4.** The data indicated that the microbial communities of the FL enrichments from AS1; AF1A; and F1 were more diverse than these of the lysine enrichments while the FL enrichment of AF4A was most similar to the lysine enrichment and that all clones were clustered with *Intestinimonas* genus (**Figure S1**). This might explain the highest butyrate formation from AF4A enrichment (Fig. 2) that more *Intestinimonas* in the FL enrichment would produce more butyrate as end metabolite. For AS1 sample, the number of sequences belonging to *Intestinimonas* genus declined substantially after the transfer to FL, which was in accordance to a small amount of the butyrate formation (Fig. 2). Only 2 of 14 clones in the FL enrichment were clustered with *Intestinimonas* spp. in AS1 while some other taxonomic groups newly appeared (**Figure S2**). Similarly, the microbiome of FL enrichment from AF1A was also highly diverse with only 3 clones within *Intestinimonas* group (**Figure S3**). There was an outgrowth of a strain that was highly similar to *Enterobacter-Klebsiella* in the FL enrichment of F1 sample that was likely due to the growth on titanium citrate, which was added as reducing agent during the incubation (**Figure S4**). As the number of clones was not high, it is possible that those related to *Intestinimonas* were not picked up. The butyrate formation from FL enrichments in all samples confirmed the activity of butyrogenic groups and the observed diverse communities in the FL lysine enrichments rather reflected the technical constraints. In addition, none of these other taxonomic groups was known as FL degrader except *Intestinimonas*. Indeed, they are known as acetogenic bacteria for instance, *Bilophila* and *Enterobacter* are known as facultative pathogenic bacteria such as *Clostridium hylemonae, Clostridium aldenense*. So those were unlikely able to degrade FL rather than sustain their survival in poor growth conditions via using some intermediates released from FL degradation by other bacteria. In conclusion, these data indicate a key role of *Intestinimonas* in FL conversion to butyrate in the intestine.

3.3. Prevalence of Intestinimonas in formula-fed infants and breast-fed infants by metagenome analysis

To the best of our knowledge, *I. butyriciproducens* is the only species that is able to degrade FL to butyrate and harbor the complete pathway for this metabolism. The FL pathway has two sub-pathways which are lysine and acetyl-CoA pathway and both lead to butyrate formation. *I. butyriciproducens* is able to use acetyl-CoA pathway when growing on glucose while it employs lysine pathway for fermenting lysine. When FL is present, this bacterium is able to use both pathway simultaneously converting 1 mole FL to 3 moles of butyrate (Bui et al., 2015). For this reason, we investigated the presence of *Intestinimonas*-derived FL and lysine pathway genes in the metagenomes of mother-infant pairs (Yassour et al., 2016, Ferretti et al., 2018). Since the number of exclusive formula-fed infants was very scarce, we included in this group the infants that received a mixture of formula and breast milk (Table 1). The influence of the FL content in mixed-fed infants to the prevalence of *Intestinimonas* might have been to a lesser extent comparing with those

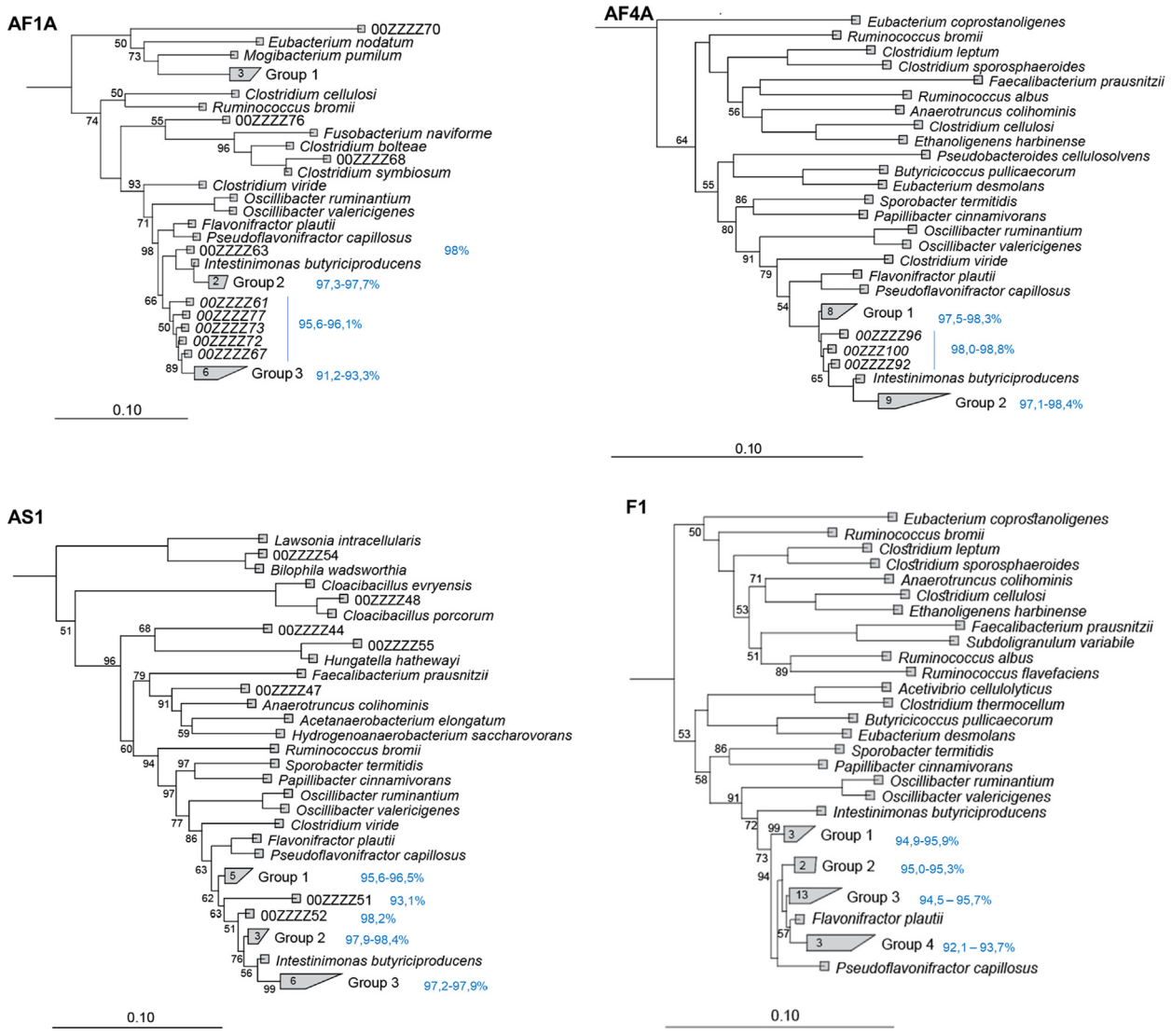


Fig. 3. Phylogenetic trees based on 16S rRNA sequences of the microbial communities of lysine enrichments from the samples F1, AF1A, AS1, AF4A and related species. A 16S rRNA sequence of *Nitrososphaera viennensis* was used as outgroup. Bootstrap values based on 1000 replications are shown at branching points. Bar, 1% sequence divergence.

Table 1
The presence of FL and lysine pathway genes in mothers and infants in the metagenome datasets from Yassour and Ferretti study (Yassour et al., 2016, Ferretti et al., 2018).

Volunteer	Total number of samples	Samples consisting FL pathway genes	Samples consisting lysine pathway genes
Yassour study			
Mother	23	22	22
Breast-fed infant	23	3	6
Mother	7	7	7
Mixed-fed infant	7	5	5
Two-sided proportionality z-test	**p-value**	0.01016	0.08331
Ferretti study			
Mother	8	6	8
Breast-fed infant	8	0	1
Mother	2	2	2
Mixed-fed infant	2	0	0

Due to the limited size of Ferretti dataset, Z test was only performed and given for Yassour data.

infants that exclusively fed with formulas. In the study of Yassour et al. (2016), the microbiome of 3 month infants including 7 mixed-fed infants (breast-fed and formula-fed) and 23 breast-fed infants was analyzed along with their mothers. Interestingly, 30 mothers were found to have FL/lysine pathway genes while only 3 breast-fed infants were detected with FL/lysine pathway out of 23 whereas 5 out of 7 mixed-fed infants were found to have FL/lysine pathway genes (Table 1). Clearly, the number of infants having FL/lysine genes out of total analyzed infants was significantly higher for those that had received formulas than that were exclusively breast-fed. It has been suggested that an infant inherits most of the microbiome from the mother (Bäckhed et al., 2015, Ferretti et al., 2018). Hence, most infants should have obtained the FL/lysine pathway genes from their mothers. However, these FL/lysine pathway genes were highly observed in the mixed-fed infants and poorly detectable in the breast-fed infants. This suggests the transfer of microbes containing the FL/lysine genes to all infants but selective outgrowth of FL/lysine-fermenting micro-organisms predominantly in the presence of formulas. These findings imply that the feeding mode and type of milk might have influenced the outgrowth of *Intestinimonas* spp. and that this species was enriched in response to FL-rich milk.

Another study of the infant microbiome from Ferretti et al. (2018) was also included in Table 1. Although 8 mothers carried the microbial

genes for the FL pathway, none of the breast-fed infants from these mothers showed the FL pathway, supporting the earlier suggestion that breast milk feeding did not facilitate the enrichment of FL-fermenting microorganisms. The FL pathway was detected in 2 mothers but absent in their mixed-fed infants.

Extrapolation of the significant results obtained with the large dataset (Yassour et al., 2016) suggests that the response of microbiota in general or *Intestinimonas* in particular to the changes of FL content in the milk given to infants might apply to the major population. This is plausible as diet has been reported as one of major factors that influenced the microbial composition in the human intestine (Flint, Duncan, Scott, & Louis, 2014). Our study gives an evident example of how intestinal microbiota responds to changes in dietary input.

4. Conclusions

The dietary components are well known as selective factor to shape the human microbiota. Intestinal bacteria also are able to efficiently cope with the changes in the diets. The concept of human gut bacteria acting as bio-remediators (Wolf et al., 2019) for indigestible dietary components attains very well to d-AGEs and the results reported in this paper on *Intestinimonas* and FL degradation are also in line with this view. Our data indicate that *Intestinimonas* plays a key role in FL degradation to butyrate in the human microbiota. The high prevalence of FL pathway genes in the microbiome of formula-fed infants but not in that of breast-fed infants indicates that FL metabolism is likely to be linked to differences in feeding modes and milk components. This was also in a good agreement with the data from the enrichments from the infants. Nevertheless, culturing studies with a larger number of infants with different feeding modes would be needed to further verify the effect of milk components on *Intestinimonas* prevalence.

5. Ethics statement

The study involved six subjects that donated fecal samples in Table 1. Informed consent was obtained from the mothers for their approximately 6 month old infants and an Asian adult, while the two African samples were derived from a previously reported intervention study and preserved in 25% glycerol (kind gift from Prof O'Keefe, Pittsburgh, PA)

6. Credit author statement

TPNB, WMdV and VF designed the experiment. TPNB conducted the experimental work and drafted the article. TPNB, WMdV, ADT and VF contributed to the interpretation of the data. GR synthesized Nε-fructosyllysine. ADT performed the measurement on Nε-fructosyllysine content in the enrichments. BN did metagenomic analysis. TPNB, WMdV, ADT and DVF provided feedback and corrections on the manuscript and approved the final version.

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Declaration of Competing Interest

The authors declare no conflict of interests

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.103974.

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