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Research Article

RESEARCH ARTICLE

Breast milk urea as a nitrogen source for urease positive *Bifidobacterium infantis*

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One sentence summary: This study indicates that nitrogen sources in human milk are potentially selecting for a healthy gut microbiome, which is important for infant feeding and health considerations.

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ABSTRACT

Human milk stimulates a health-promoting gut microbiome in infants. However, it is unclear how the microbiota salvages and processes its required nitrogen from breast milk. Human milk nitrogen sources such as urea could contribute to the composition of this early life microbiome. Urea is abundant in human milk, representing a large part of the non-protein nitrogen (NPN). We found that *B. longum* subsp. *infantis* (ATCC17930) can use urea as a main source of nitrogen for growth in synthetic medium and enzyme activity was induced by the presence of urea in the medium. We furthermore confirmed the expression of both urease protein subunits and accessory proteins of *B. longum* subsp. *infantis* through proteomics. To the same end, metagenome data were mined for urease-related genes. It was found that the breastfed infant's microbiome possessed more urease-related genes than formula fed infants (51.4:22.1; 2.3-fold increase). *Bifidobacteria* provided a total of 106 of urease subunit alpha alignments, found only in breastfed infants. These experiments show how an important gut commensal that colonizes the infant intestine can metabolize urea. The results presented herein further indicate how dietary nitrogen can determine bacterial metabolism in the neonate gut and shape the overall microbiome.

Keywords: urease; infant gut microbiota; *Bifidobacterium;* urea; human milk

Importance paragraph

Human milk stimulates a health-promoting microbiome in infants. Urea is abundant in human milk, making up a large part of the non-protein nitrogen. We wanted to explore if human milk urea could be used by the microbiota. Considering bacterial nitrogen metabolism for further understanding why breastfeeding is so beneficial, is the next step. In that fashion, this study indicates that nitrogen sources in human milk are potentially selecting for a healthy gut microbiome, which is important for infant feeding and health considerations.

INTRODUCTION

When human life begins, the gut microbiota develops dynamically (Yatsunenko *et al.* 2012; Backhed *et al.* 2015). Notably,

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this microbiota can aid with digestion and thus supports the infant's nutritional needs (Koropatkin, Cameron and Martens 2012; Fernández *et al.* 2013; LeBlanc *et al.* 2013). Moreover, the microbiome is crucial in establishing health for the infant by supplying amino acids and vitamins and contributing to gut maturation and immune development (Fuller and Reeds 1998; Martin *et al.* 2010; Azad *et al.* 2014; Wopereis *et al.* 2014; Koleva, Bridgman and Kozyrskyj 2015; Neis, Dejong and Rensen 2015; Gensollen *et al.* 2016). This vital balance originates in the early moments of life and the bacteria responsible are promoted by dietary factors named prebiotics (Backhed *et al.* 2015; Goldsmith *et al.* 2015; Tanaka and Nakayama 2017). In recent years, research has therefore focused on describing early microbial colonization patterns and mechanisms of human milk utilization by early life gut symbionts (Sela 2011; Marcobal and Sonnenburg 2012; Milani *et al.* 2017; Shani *et al.* 2018). Human milk and all of its components are likely to have evolved to promote symbiosis between host and microbiome. However, it is currently unclear how the microbiota salvages nitrogen from breast milk. Human milk nitrogen comes in many forms and is for example represented by milk proteins, peptides and free amino acids, but also human milk oligosaccharides hold nitrogen that could be utilized by the infant gut microbiota (Harzer, Franzke and Bindels 1984; Carratù *et al.* 2003; Asakuma *et al.* 2011; Sela 2011; Ballard and Morrow 2013; Andreas, Kampmann and Mehring Le-Doare 2015; James *et al.* 2016; Sakanaka *et al.* 2019). Interestingly, this complex bio-fluid holds some nitrogen sources that are waste products of human metabolism, like urea (Carlson 1985; Donovan and Lonnerdal 1985; Andreas, Kampmann and Mehring Le-Doare 2015).

Urease is the first enzyme ever isolated (Sumner 1926). As such, the enzyme urease (*EC* 3.5.1.5) has been studied in various bacteria and ecosystems and has been found to play a crucial role in bacterial survival (Burne and Chen 2000; Scott *et al.* 2002; Mora and Arioli 2014). Urease is a nickel or iron co-factored multimeric enzyme that catalyses the hydrolysis of urea into carbon dioxide and ammonia (NH_3; Zimmer 2000; Quiroz-Valenzuela *et al.* 2008; Carter *et al.* 2011). In bacterial pathogens, e.g. *Helicobacter pylori*, *Proteus mirabilis*, urease functions as a virulence factor (Dupuy *et al.* 1997; Hola, Peroutkova and Ruzicka 2012; Mora and Arioli 2014; Roesler, Rabelo-Gonçalves and Zeitune 2014). It functions as such by managing the environment around the bacterial cell, through neutralization of the acidic microenvironment by protonation of ammonia, resulting in ammonium (NH_4) (Scott *et al.* 2002; Fu *et al.* 2018). Ureolytic activity is seen with non-pathogenic bacteria as well (Suzuki *et al.* 1979; Mora and Arioli 2014). However, in both pathogens and non-pathogens the underlying mechanism and ecological function are often unclear (Burne and Chen 2000; Mora and Arioli 2014; Lerm *et al.* 2018; Tarsia *et al.* 2018). The resulting ammonium has been suggested as the main nitrogen source of choice for amino acid synthesis by gut bacteria through glutamate dehydrogenase activity (Belzer *et al.* 2005; Stanley 2009; Davila *et al.* 2013; Neis, Dejong and Rensen 2015). Therefore, bacterial urease can fulfil a function in gut nitrogen cycling to serve the bacterial needs.

Nitrogen availability, specifically urea, both host- and diet-derived, can greatly affect the human gut microbiota (Brown, Hill and Richards 1971; Jackson 1994; Wong *et al.* 2014; Holmes *et al.* 2017; Reese *et al.* 2018). For diet, the biological norm is that for approximately the first 6 months of human life, infant feeding consists solely of human milk because of its effect on microbiota composition and overall health (Kramer and Kakuma 2004; Rinne *et al.* 2005; Backhed *et al.* 2015; Tanaka and Nakayama 2017; Stewart *et al.* 2018). Human milk composition varies inter-individually and over time from colostrum to late lactation (Ballard and Morrow 2013). It holds a collection of non-protein nitrogen (NPN) sources (25% of total N), including urea (Carlson 1985; Janas, Picciano and Hatch 1985; DONOVAN and Lonnerdal 1989; Ballard and Morrow 2013; Andreas, Kampmann and Mehring Le-Doare 2015). Urea constitutes 15% (3–6 mM) of this NPN, while others claim that it actually represents 15% of total nitrogen in human milk (Harzer, Franzke and Bindels 1984; Neville *et al.* 1984; Donovan and Lonnerdal 1985; DONOVAN and Lonnerdal 1989; George *et al.* 1996; Smilowitz *et al.* 2013; Mora and Arioli 2014). The relevance of urea nitrogen salvation for the infant gut, although not intensively studied, has often been suggested (Harzer, Franzke and Bindels 1984; Heine, Tiess and Wutzke 1986; Fomon *et al.* 1987; Fuller and Reeds 1998; Sela *et al.* 2008; George *et al.* 1996). There are, nonetheless, a few indications of urease activity by bacteria in the infant lower gut (Crociani and Matteuzzi 1982; Heine *et al.* 1984; Meakins and Jackson 1996; Millward *et al.* 2000). In an early study, labelled 15N urea turned up in microbial protein and in infant serum amino acids, indicating a function of urea in microbial biosynthetic processes and showing utilization out of a dietary source (Heine *et al.* 1984; Millward *et al.* 2000). Notably, in an infant fed with a breast milk-like diet, utilization of urea increased significantly compared to the infants fed with formula (George *et al.* 1996). Finally, a recent metaproteomics study showed enrichment of nickel transport systems in the infant gut assigned to *Bifidobacteriaceae*, which are potentially important for activation of urea metabolism (Cerdó *et al.* 2018).

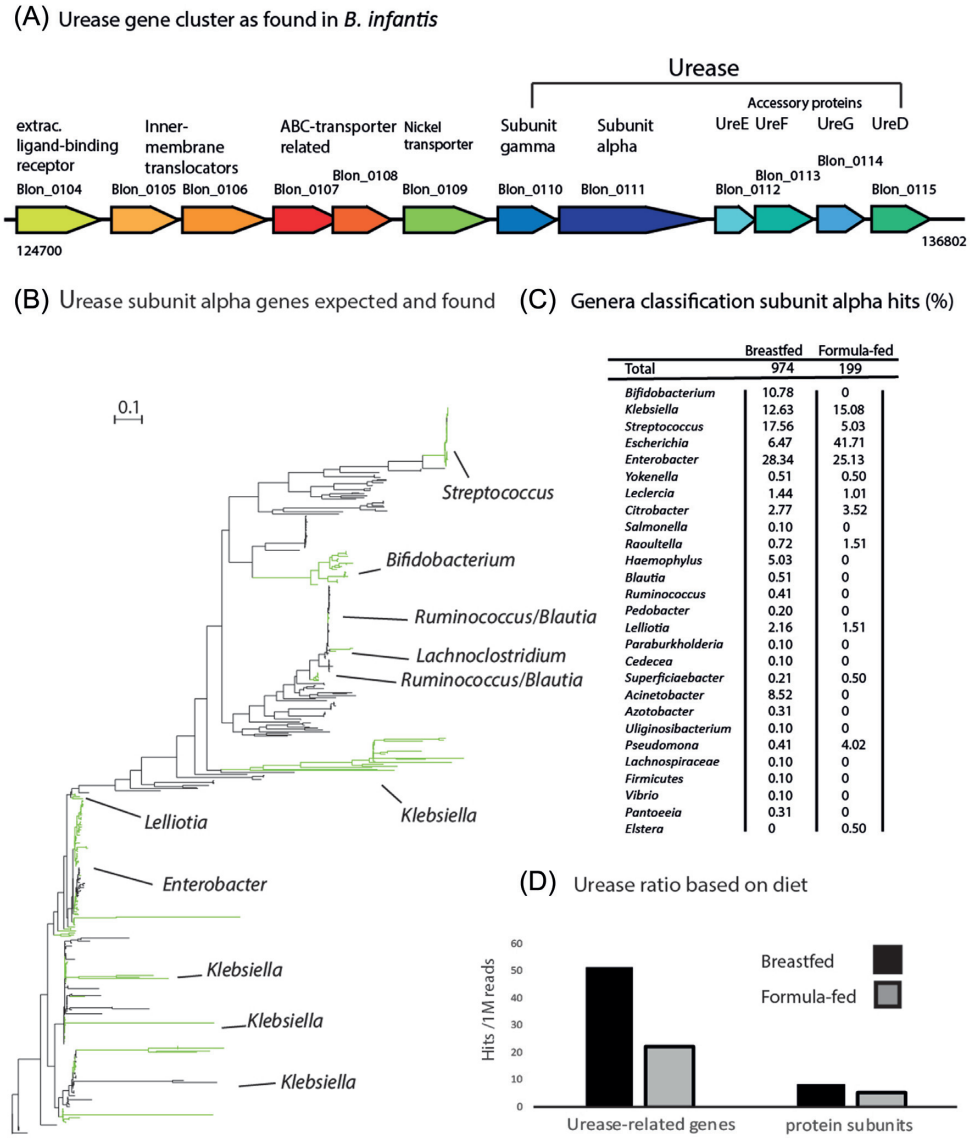
Early studies, observed urea degradation among gut commensals without further characterization of the process (Suzuki *et al.* 1979; Crociani and Matteuzzi 1982). Interestingly, several genomes of *Bifidobacterium* spp. possess urease genes (Sela *et al.* 2008; LoCascio *et al.* 2010). Of particular interest was the discovery of a full urease cluster in the common infant gut colonizer *Bifidobacterium longum* subsp. *infantis* (*B. infantis*, ATCC15697; *Blon_0104-Blon_0115*, Fig. 1A; Sela *et al.* 2008). As biomarkers for infant health, urease is likely to fulfil a different niche function for *Bifidobacterium*, compared to a function in virulence in model organisms. It is hypothesized here that *B. longum* subsp. *infantis* urease is a growth factor for the species.

In this paper, we further characterized urease activity as a growth factor *in vitro* for *B. longum subsp. infantis*, a potentially beneficial bacteria and a common infant gut colonizer (Sela *et al.* 2008; Underwood *et al.* 2015; Stewart *et al.* 2018). This specific trait might explain why *B. infantis* is an efficient colonizer of the infant gut and how it is adapted to the human milk diet. Public metagenome data of the infant gut was studied to investigate the relationship between diet and potential urease activity of the microbiome. We expect *Bifidobacterium* to interact with human milk urea. Both aspects increase our understanding of how breastfeeding might stimulate a beneficial microbiome.

MATERIALS AND METHODS

Metagenome mining for urease genes

An infant gut metagenome database comprised of three publicly available datasets along with dietary metadata was studied for occurrence of urease-related genes (Schwartz *et al.* 2012; Yatsunenko *et al.* 2012; Vallès *et al.* 2014). The study holds a total of 92 samples obtained from 74 infants with a total of 10 442 761 DNA reads (Figure S1 and S2, Supporting Information). These three studies were selected since they all contain both



	Breastfed	Formula-fed
Total	974	199
Bifidobacterium	10.78	0
Klebsiella	12.63	15.08
Streptococcus	17.56	5.03
Escherichia	6.47	41.71
Enterobacter	28.34	25.13
Yokenella	0.51	0.50
Leclercia	1.44	1.01
Citrobacter	2.77	3.52
Salmonella	0.10	0
Raoultella	0.72	1.51
Haemophylus	5.03	0
Blautia	0.51	0
Ruminococcus	0.41	0
Pedobacter	0.20	0
Lelliotia	2.16	1.51
Paraburkholderia	0.10	0
Cedecea	0.10	0
Superficiaebacter	0.21	0.50
Acinetobacter	8.52	0
Azotobacter	0.31	0
Uliginosibacterium	0.10	0
Pseudomona	0.41	4.02
Lachnospiraceae	0.10	0
Firmicutes	0.10	0
Vibrio	0.10	0
Pantoeeia	0.31	0
Elstera	0	0.50

Figure 1. (A). Urease gene cluster as found in *B. infantis* (Sela *et al.* 2008) [58]. **(B)**. Phylogenetic tree of urease subunit alpha genes expected in the human gut. Nodes of genes found in study labeled green. **(C)**. Genera of origin of the urease gene hits in the metagenomes, retrieved from all urease protein subunit alpha hits surpassing the alignment quality threshold (%). **(D)**. Normalized ratio of reads that aligned with urease proteins for all bacterial species based on infant diet (per 1 million reads).

breastfed and formula-fed infants. Included infants were solely breastfed or solely formula-fed and were 6 months or younger. Antibiotic-treated, diseased or pre-term infants were excluded from the study. The reads were previously quality filtered in different methods by the authors of before mentioned publications. To extract predictive functional data from metagenomes, protein aligners have been developed (e.g. DIAMOND; Buchfink, Xie and Huson 2015; Westbrook *et al.* 2017). Using DIAMOND, the raw shotgun reads were translated into protein and mapped against UniProt databases (Swiss-Prot and TrEMBL, Release 2019_01), to create a functional profile. The complete UniProt database was in this case included to counter the lack of inclusions of *Bifidobacterium* protein sequences and genes in the SwissProt database alone. Quality cut-offs for DIAMOND alignment were set at 60% coverage of the read length and 60% alignment identity for the protein alignments. Alignment results were filtered for urease-related hits. These included urease protein subunits, urea transporters, urease accessory proteins and urease activity regulatory genes. Protein hits were normalized per 1 million high quality reads. This method yielded occasionally multiple successful assignments per read. Hits with the highest reported identity scores were considered for taxonomic analysis. One sample from the Schwartz *et al.* (2012) dataset was excluded since it contained a far higher rate of urease genes than the other samples combined from that study. We suspect an artefact resulting from our methods or that the infant was suffering from an infection, which warranted exclusion. Included samples list and dietary metadata are available in the supplementary data (Table S1, Supporting Information). A phylogenetic tree for urease subunit alpha genes related to the infant gut was constructed using ARB software package (max. likelihood; Phylip PROML), based on the 25 most dominant taxa in study by Stewart *et al.* (2018) and Ludwig *et al.* (2004). Original tree file was uploaded at Open Science Framework repository (OSF, https://osf.io/2hu4m). From NCBI Nucleotide database, full genome sequence annotations of *Bifidobacterium* were checked for urease gene clusters.

Bacterial strains and growth conditions

Bifidobacterium longum subsp. *infantis* (ATCC 17930/DSM20218/JCM1260) and *Bifidobacterium breve* (ATCC 15698/DSM20091) were anaerobically grown on TOS-propionate agar medium (Sigma-Aldrich, St. Louis, USA), specialized for enumeration of *Bifidobacterium* species. Plated cultures were incubated at 37°C and plates were stored at 4°C for long-term storage. Plates cultures were used to inoculate liquid pre-cultures by swiping colonies in an anaerobic tent (Coy Vinyl Anaerobic Chamber, max. 5% H_2/N_2). For liquid culturing, a nitrogen-limiting medium with excess in carbon source (lactose 2%; pH 6.2): yeast extract (1 g/L); potassium dihydrogen phosphate (3 g/L); dipotassium hydrogen phosphate (4.8 g/L), magnesium sulphate (0.2 g/L), sodium propionate (15 g/L) and L-cysteine hydrochloride (0.5 g/L) to counter the strain's auxotrophy for the amino acid. Serum bottles were supplemented with filter-sterilized vitamin mix and trace metals (100x; originally designed for *Lactobacillus lactis* (Otto *et al.* 1983)). Nitrogen treatments included urea (0.6 g/L; 10 mM), tryptone, (protein digest, w/w: 12.7% N, 2.2 g/L, Oxoid, Basingstoke, UK), ammonium sulfate ($(NH_4)_2SO_4$) or no added nitrogen source. Nickel chloride (NiCl; 0.1–1 mM) was included as it is required by active urease enzyme. Test cultures were subsequently inoculated with 0.1 mL of late logarhytmic phase pre-culture (24 h). Anaerobic serum bottles possessed either a headspace of N_2 or CO_2/N_2 (80%/20%) at 1.7 atm. The basal growth media was tested in a pH range and with a bicarbonate buffer (CO_2/N_2; 20 mM Na_2CO_3). Liquid pre-cultures contained only CO_2/N_2. Growth was evaluated by measuring optical density at 600 nm (OD 600; OD 600 DiluPhotometer, IMPLEN, Germany). Acidification was observed by measuring pH (ProLine B210). For statistical analysis, an unpaired *t*-test was used in SPSS Software (V24). *P* values < 0.05 were considered statistically significant.

Urea colorimetric assay

Urea levels were determined by a colorimetric urea assay (MAK006; SigmaAldrich) according to manufacturer's instruction. The colored result of a coupled enzyme reaction was measured at 570 nm on a Synergy Mx microplate reader (Biotek, Winooski, USA). Culture supernatants were diluted 100x prior to analysis to fit the kit's standard curve. Data were normalized to observed background effects due to interference of compounds in the media, like e.g. ammonium, $NAD^+/NADP^+$ and pyruvate.

Urease enzymatic assay

Bacterial cultures (1 mL) were retrieved and centrifuged (10 min, 20 000 x *g*) before supernatants were separated from pellets. Cell pellets were suspended in sodium phosphate buffer (10 mM) before analysis. Supernatants were filtered (0.2 μm) before use in enzymatic assay. Sonication of bacterial cell pellets was performed using a MS72 probe on a Bandelin Sonopuls Sonicator (Bandelin Electronic, Berlin, Germany). Cells were lysed using 20 s sonication at 30% amplitude with 30 s intervals on ice and repeating this four times. Enzymatic activity of supernatants and cell lysates was then assessed using urease activity assay kit (MAK120, Sigma Aldrich) as reported by the manufacturer, derived from the Berthelot method. Exposure to urea lasted for 10 min. After 30 min of color formation, absorbance at 670 nm was measured, preceded by 2 s medium shake on a Synergy Mx microplate reader (Biotek).

Proteomics

Samples were taken from mono-cultures of *B. infantis* (ATCC17930) with urea during late exponential growth phase. Cell lysis was achieved through bead beating in a FastPrep-24 5G instrument (MP Biomedicals, Brussels, Belgium) for six times 30 s at 6.5 m/s with cooling after every bead step. Protein concentration was assessed using Pierce BCA protein assay (ThermoFisher Scientific, Waltham, MA; Figure S8, Supporting Information). Subsequent protein digestion was performed overnight using dithiothreitol (DTT, 2 mM), iodoacetamide (IAA, 4 mM) and trypsin (1:50 of a 1 μg/μL solution) at 37°C. Cleanup was performed through SPE columns (Solid Phase Extraction; ThermoFisher Scientific) with acetic acid (100 mM in 95% acetonitrile) to be finally dissolved in the eluent ammonium formate (10 mM). Samples were analyzed in duplicate by nano-LC-HRMS/MS as described by Meiring *et al.* (2002). An Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, USA) was used, connected to a Q-Exactive Plus Mass spectrometer (ThermoFisher Scientific). Peptides were trapped on a 100 μm inner diameter trap column packed using ReproSil-Pur C18-AQ, 3 μm resin (Dr. Maisch, Ammerbuch, Germany) at 5 μL/min in 100 mM acetic acid. Afterwards the peptides were eluted at 100 mL/min in a 90 min extended gradient from 10 to 40% acetic acid solvent (in 95% acetonitrile) to a 20-cm IntegraFrit column (50 μm inner diameter, Reprosil-Pur C18-AQ 3 μm, New Objective, Woburn, USA). The acquired spectra were analyzed using Thermo Proteome Discoverer in combination with Mascot (ThermoFisher Scientific). The reference database comprised of protein sequences from *B. longum* subsp. *infantis from Uniprot* (ATCC15697) since the genome of *B. infantis* strain ATCC17930 is not publically available.

RESULTS

Occurrence of urease gene cluster in *Bifidobacterium* spp.

Bifidobacterium whole genome annotations were scanned for urease gene clusters as present in *B. infantis* (Fig. 1A; Sela, 2011). A total of five *Bifidobacterium* urease genes are currently annotated across seven species that are related to the human gut. Namely, the *B. longum* subspecies *longum*, *infantis* and *suis*; *B. subtile*; *B. kashiwanohense* and *B. scardovii* (Table S3, Supporting Information). Either a full gene cluster or no gene cluster was found to be present every time. One exception occurred with *B. subtile*, where one accessory protein gene was missing from the cluster. Interestingly, of *B. infantis* whole genome sequences only 50% of the cases had a urease gene cluster annotated (Table S3, Supporting Information). Meanwhile, no urease gene clusters are currently annotated in several other common *Bifidobacterium* infant gut colonizers like *B. breve* and *B. bifidum* spp.

Urease genes found in infant gut metagenomes

We studied bacterial urease gene cluster occurrence in the infant gut of which *B. infantis* holds an example (Fig. 1A). A phylogenetic tree was constructed of urease alpha subunit genes that are expected to occur in the infant (Fig. 1B). Interestingly, all *Bifidobacterium* genes cluster together. Furthermore, many genes, belonging to other genera have been traced to the metagenomes included in this study (Fig. 1B, green highlights). In total, the functional profile of the metagenomes yielded a total of 27 taxa

for urease protein subunit alpha hits (Fig. 1C). The most dominant genus holding urease genes was *Enterobacter*, representing 27.8% (326) of alignments. The other most dominant found genera were *Streptococcus* (15.4%), *Klebsiella* (13%), *Escherichia* (12.4%). *Bifidobacterium* was the fifth most abundant genus with 9% (Fig. 1C). The genus was represented by *B. longum* subspp., *B. kashiwanohense* and *B. scardovii*. Interestingly, *Bifidobacterium* urease genes were only found in breastfed infants, while *Escherichia* hits occurred mostly in samples belonging to strictly formula fed infants (breastfed 6%, formula-fed 41%). Sequences traced to *Enterobacter* were present in both diet types 28.34% and 25.13% in breastfed and formula-fed infants respectively. *Klebsiella* showed a similar pattern (12.63%; 15.08%). After data normalization a 2.3-fold increase of urease-related hits was observed for breastfed infants compared to formula-fed infants (Fig. 1D). A 1.5-fold increase was observed when measuring urease protein subunits separately.

Urea serves as a nitrogen source for *B. longum* subsp. *infantis*

We tested the ability of *B. infantis* to use urea as the main nitrogen source by comparing it with growth on a protein digest (tryptone) or no added nitrogen. Growth on urea as the main nitrogen source was observed for *B. infantis* and not for *B. breve*, which was included as a non-urease expressing control (Fig. 2A and B, 0.4 compared to 4.1; OD 600; 32 h; $P \leq 0.05$, Figure S4 and S5, Supporting Information). Differences in growth were reflected by different pH across the culture types (Figure S4, Supporting Information). Growth on $(NH_4)_2SO_4$ was checked to confirm the strain's ability to process the ammonium output resulting from degrading urea (Figure S7, Supporting Information; headspace: N_2). An active urease complex produces carbon dioxide (CO_2).

Growth was therefore compared to conditions with CO_2 present. Growth increased in the presence of CO_2 compared to conditions with only N_2 present, but the presence of urea still increased the growth of *B. infantis* relative to the basal medium (Fig. 2B). Thus, CO_2 absence is not a trigger for *B. infantis* to become urease active.

Urease proteins confirmed and activity is linked to the presence of urea

Presence of urease-related proteins was confirmed with high reliability for several proteins deriving from the *B. infantis* urease gene cluster (Figure S8, Supporting Information). This included urease subunit alpha, beta/gamma and accessory proteins UreE and UreG (Fig. 1A and Figure S8, Supporting Information). This thus included Uniprot accessions: B7GT16-18 and 20. The sequence coverage can be considered low in some cases (4–35%). However, the protein identification can be regarded as very confident due to the occurrence of unique peptide sequences (3–7) and the additional spectral evaluation performed by the Proteome Discoverer software (Figure S8, Supporting Information). Urease activity of *B. infantis*, was only detected upon cultivation with urea as the nitrogen source (Fig. 3, $P < 0.05$, and Figure S5, Supporting Information). At 20 h no activity was observed in basal medium and basal medium supplemented with tryptone. No clear and reliable activity measurements were obtained, when the supernatant was analyzed. Degradation of 10 mM urea was confirmed at 24 h, which corresponds with the exponential growth phase (Figure S6, Supporting Information).

DISCUSSION

Since the discovery of a urease gene cluster in *B. infantis* (Sela *et al.* 2008), urea nitrogen salvaging by the infant gut microbiota was highlighted as of potential importance to the settling microbiota. Our results indicate once more, the adaptation of *B. infantis* towards the human milk diet and gives rise to speculation on the role of urea in breastfeeding. Together with the metagenome data this study illustrates a new mechanism by which urea in breast milk can shape a microbial community and thus strengthens the hypothesis that urease could be used as a growth factor for a potentially beneficial strain.

Metagenome study

Bifidobacterium urease represents a significant part of the microbiota that is potentially utilizing urea in breastfed infants (Fig. 1B and C). Notably, some *Bifidobacterium* hits belonged to *Bifidobacterium callitrichos*, *Bifidobacterium primatium*, *Bifidobacterium biavatii* and *Bifidobacterium tissieri* that are not associated with the human infant gut, but rather with the primate gut that is likely to have urea available as well (Endo *et al.* 2012; Michelini *et al.* 2016; Modesto *et al.* 2018). This indicates that there might be more *Bifidobacterium* urease genes associated with the human infant gut that are not described. The constructed tree shows a likelihood of alternate evolutionary paths of urease activity due to large spreading within phylogenetic groups, while in the meantime *Bifidobacterium* clustered together and thus showing similarity. The functional profile showed that *Streptococcus* urease genes are dominantly found in the breastfed infant (Fig. 1C) and their urease activity is connected to biosynthetic pathways and provides intracellular benefits due to pH regulation (Arioli *et al.* 2007; Arioli *et al.* 2010; Mora and Arioli 2014). It can now be hypothesized that *Bifidobacterium* urease fulfills a similar role, especially considering the highly acidic environment that is the infant colon. Interestingly *Escherichia* (*E. coli*) shows a reverse pattern compared to *Bifidobacterium*. The genus appeared more in formula-fed infants compared to breastfed infants, while their urease activity has been mostly associated with pathogenesis (Konieczna *et al.* 2012). However, this might be due to the lack of prebiotics in the formula products, of which no data was available. Moreover, several other pathogenic genera, e.g. *Citrobacter* and *Klebsiella* were found (Rae, Fazio and Rosales 1991; Tumbarello *et al.* 2015; Pal and Mahendra 2016). Urea metabolism has been intensively studied for these pathogenic genera, which could result in an underrepresentation of *Bifidobacterium* urease proteins in the database, a common bias and obstacle when constructing functional profiles (Quince *et al.* 2017). Also, *Bifidobacterium* spp. have one locus containing an urease gene cluster annotated, while others like *Escherichia coli* can have multiple (Heimer *et al.* 2002). Proper validation or normalization for such biases, should be developed. Nonetheless, beneficial bacteria, e.g. *B. infantis*, that possess urease activity might compete with pathogens for urea as a nitrogen source. Bifidobacteria might be dominant in breastfed infants due to their capacity to degrade HMOs and through this mechanism, it could limit the possibility of other urease positive species occurring in that same environment. Moreover, it might be acting as a detoxifier, since urea clearance from the gut is important in gut health (Ramezani *et al.* 2016). This could make *Bifidobacterium* Urease a target for therapeutics. How dietary urea would favor *B. infantis* or other *Bifidobacterium* spp. over these potential pathobionts needs to be further investigated.

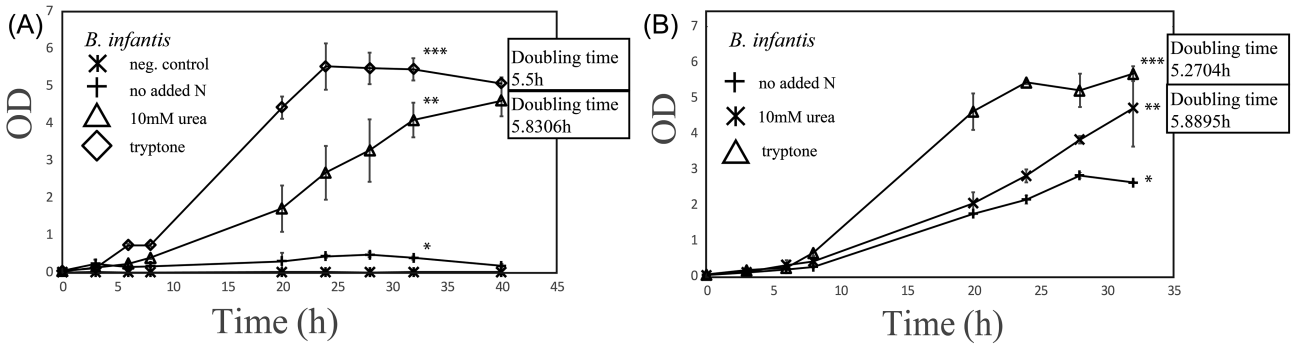


Figure 2. (A). Growth of *B. infantis* in nitrogen limiting media, headspace 100% N_2 (OD 600). **(B)**. Growth of *B. infantis* in nitrogen limiting media, headspace 80% N_2/CO_2, * *P* values < 0.05. Doubling time in minutes.

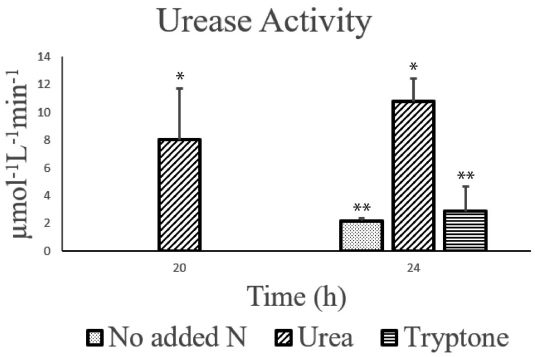


Figure 3. Urease enzyme activity (ammonium production/µmol/L/min) measured on two timepoints with the Berthelot method, $P < 0.05$ (Figure S5, Supporting Information).

The functional profile showed that breastfeeding selects for genes related in urea metabolism and thus bacteria involved in urea utilization (Fig. 1D). However, this pattern was not observed consistently for urease protein subunits when looking at the included studies separately (Figure S2, Supporting Information). This data nonetheless provides a fair representation of the total level of genomic capabilities for a specific process, compared to an approach that included assembly or binning. Short genetic fragments of low abundance species that lack sufficient sequence coverage are now included because of recent advances in fast translated searches (Buchfink, Xie and Huson 2015; Quince *et al.* 2017; Franzosa *et al.* 2018).

Urea in the infant gut

It is expected that urea is a microbiome modulator in the infant gut and that urease producing microbes profit in a comparable way as for example how host-derived urea in uremic patients selects for urease active bacteria (Brown, Hill and Richards 1971; Wong *et al.* 2014). Urea could be present at low levels in the lower gastrointestinal tract of the infant due to earlier ureolytic activity along the gastrointestinal tract or because of inter-individual variation between maternal factors that affect milk composition (Harzer, Franzke and Bindels 1984; Fuller and Reeds 1998; Montecucco and Rappuoli 2001; Ballard and Morrow 2013; Mora and Arioli 2014; Andreas, Kampmann and Mehring Le-Doare 2015). Urea can be supplied by the human body, or by other bacteria through amino acid fermentation as well (Crociani and Matteuzzi 1982; Heine *et al.* 1984; Meakins and Jackson 1996; Fuller and Reeds 1998; Millward *et al.* 2000; Davila *et al.* 2013; Mora and Arioli 2014; Neis, Dejong and Rensen 2015). Notably, lower protein diets have been shown to change urea kinetics in adults, shifting towards more host-derived urea utilization (Langran *et al.* 1992; Meakins and Jackson 1996). A similar pattern could exist for the infant gut. Host-derived urea can anyhow explain the level of urease genes found in formula-fed reference group. Moreover, infants fed formula were not completely excluded from dietary urea, since it occurs in formula as a trace compound due to its cow milk origin (DONOVAN and Lonnerdal 1989). The hypothesis that milk urea serves a biological purpose through possibly selecting for specific bacteria, is nonetheless underlined by the described results.

Urea utilization by *B. infantis*

We provide evidence for the ability of *B. infantis* (ATCC17930) to utilize urea as a main nitrogen source. The strain is able to express necessary gene products, which were confirmed through proteomics albeit with low coverage. The low coverage may be explained by the use of the related proteome database, which likely contains only homologue sequences of the targeted enzyme. While being comprised of fairly large proteins, which can lower coverage on its own, it is expected that the urease enzyme is embedded in the membrane (Bode *et al.* 1993). This might lead to lesser efficiency with the described protein extraction method due to enzyme being lost with the cell debris. The likelihood of the strain being able to produce active enzyme, was confirmed by the presence of the accessory activator UreE in the proteomics data (Quiroz-Valenzuela *et al.* 2008). This accessory protein is thought to be involved in nickel-chelation which is essential for urease activation in other bacteria (Lee *et al.* 1993; Song *et al.* 2001). The lack of UreD and UreF occurrence cannot be explained, especially since all other data from this study suggests urease activity in *B. infantis*. Possibly this accessory protein is not required for active enzyme or it is produced in another stage of the culture experiment. Accessory protein UreF was scarcely detected, but never surpassed the low reliability threshold. Interestingly, according to enzymatic assay, only the presence of urea made *B. infantis* active in degrading urea, which indicates some form of metabolic switching and regulation according to the enzymatic assays (Fig. 3A). Similar observations were made for other bacteria hosted by humans that have proven to be urease active and that specifically transcription was regulated by UreR in the presence of urea (Poore and Mobley 2003; Belzer *et al.* 2005). The fact that the presence of urea is required for *B. infantis* was furthermore observed in *Streptococcus thermophilus*, while also indicating that aspartate metabolism and glutamate metabolism might be linked to urease activity (Arioli *et al.* 2007; Zotta *et al.* 2008). Since we tested urease activity in pure strain

cultures, there is a chance that the organism changes it activity in the presence of other species in a community. Further investigation into the regulation of the urease gene cluster in *B. infantis* is therefore definitively warranted. Due to presence of genes encoding a transporter system on the cluster (Fig. 1A), it is to be expected urease activity is at least partially intracellularly and thus related to the cell lysate as was shown in this study. Urease activity in the supernatant has not been reliably measured using the enzymatic assay, but cannot be excluded. It has been shown that CO_2 availability promotes growth of *B. infantis*, yet lack of CO_2 is not a trigger to become ureolytic in this organism, since growth occurs under both conditions. The promoting effect of CO_2 indicates the capnophilic nature of this organism. Despite the importance of Bifidobacteria for infant health, the effect of CO_2 on the overall metabolism of the genus is poorly investigated and is surely related to the effect of urease activity by these bacteria. Urease activity can furthermore be a means of pH regulation by intestinal bacteria for the acidic environment that the breastfed infant's gut traditionally is (Scott *et al.* 2002; Arioli *et al.* 2010; Fu *et al.* 2018; Henrick *et al.* 2018). These results cannot exclude that *B. infantis* benefits from urease activity in low pH and even regulates the pH (Figure S4, Supporting Information).

Conclusive words

Human milk shapes our microbiome from the moment we are born. The authors hypothesize that urea in breast milk functions as an important nitrogen source for beneficial bacteria in the early life gut microbiota and provide the first evidence for this. To our knowledge this is the first time that a direct link has been made between *Bifidobacterium* and urease activity and meanwhile its relevance in the infant gut is supported by metagenomic data. This study further characterizes a mechanism by which a common gut symbiont utilizes urea as a nitrogen source from human milk. It is proposed here that urea degradation by *B. infantis* is another relevant means to acquire nutrients from breast milk. Further research is needed to clarify the function of *B. infantis* urease activity in the infant gut ecosystem.

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The study was designed by P.S., L.K., R.B., J.K. and C.B.; P.S. performed the study in the lab; P.S. collected and generated the data from the metagenomes; P.S. performed the proteomics study; P.S. performed data analysis and figure generation; P.S. and C.B. wrote the manuscript. The manuscript was checked by P.S. L.K., R.B., J.K. and C.B. All authors contributed to critical revisions and approved the manuscript. First of all, the authors would like to thank Dr Bernd Stahl[2] for being an initial inspiration to this project. Secondly, the authors would like to thank Athul Sundaresan[1] for his contributions in the lab as part of his thesis project. Finally, the authors would finally like to thank Heleen de Weerd[2], Joost Gouw[2] and Gido Jehoel[2] for their help and advice with the metagenomics data generation, proteomics processing and analysis.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

Conflicts of interest. R.B. and J.K. are employees of Danone Nutricia Research. P.S., L.K., C.B. received funding from Danone Nutricia Research.

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