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N-glycans from human milk glycoproteins are selectively released by an infant gut symbiont *in vivo*

Sercan Karav[a], Giorgio Casaburi[b], Aysenur Arslan[a], Merve Kaplan[a], Berfin Sucu[a], Steven Frese[b,c,*]

[a] Department of Molecular Biology and Genetics, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
[b] Evolve Biosystems, Inc, Davis, CA 95618, USA
[c] Department of Food Science and Technology, University of Nebraska, Lincoln, Lincoln, NE 68588, USA

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ABSTRACT

Complex, indigestible free oligosaccharides as well as conjugated glycans are found in milk that shape the gut microbiome of infants. The activity of an endo-β-*N*-acetylglucosaminidase from *B. longum* subsp. *infantis* (*B. infantis*) is known to release *N*-glycans from native milk glycoproteins under physiological conditions. We investigated whether this enzyme is active *in vivo* in breastfed infants fed *B. infantis* EVC001. Using mass spectrometry, we found 19 *N*-glycans related to human milk glycoproteins increased in abundance, similar to previous work using bovine milk glycoproteins, and these 19 *N*-glycans matched unique specificities of this enzyme. Twenty *N*-glycans were unique to infants fed *B. infantis* EVC001. *Bifidobacteriaceae* were correlated with these glycans, confirming the relationship between *B. infantis* and released *N*-glycans. This suggests that this enzyme is active *in vivo* and releases *N*-glycans from milk glycoproteins, and may play a role in *B. infantis* EVC001 colonization of the gut microbiome.

1. Introduction

Millenia of evolution have shaped human milk into the nutritionally ideal food for infants. Stringent selective pressures have shaped the components of human milk to maximize their functional and nutritional utility (Garrido et al., 2013). Apart from containing protein, fat, and carbohydrates that feed the infant, human milk contains a high concentration of oligosaccharides ranging in complexity and composition (Zivkovic et al., 2011). These oligosaccharides (human milk oligosaccharides, HMOs) represent a major fraction of the total solid fraction of human milk, though the exact concentration varies across lactation and between individuals (Smilowitz et al., 2013; Xu et al., 2017). HMOs are selectively utilized by members of the infant gut microbiome, predominantly by *Bacteroides* and infant-associated *Bifidobacterium,* and uniquely by *B. infantis* (Garrido et al., 2012; LoCascio et al., 2007; Matsuki et al., 2016; Sela et al., 2008).

In addition to HMOs, glycans are also found in conjugated forms, bound to either lipids or proteins. *N*- and *O*-linked glycans conjugated to proteins in human milk may represent a consequential fraction of complex carbohydrates that is selectively accessible by members of the distal gut microbiome. The similarity in composition and structural linkages between HMOs and glycans has led to interest in the functional role of these bound glycans in modulating the gut microbiome (Garrido et al., 2016). Recent work has shown that the *N*-glycans bound to milk glycoproteins such as lactoferrin, and immunoglobulins facilitate the growth of *Bifidobacterium longum* subsp. *infantis* (*B. infantis*; Karav et al., 2016). Further, these glycans can be released from glycoproteins by the action of a unique enzyme, endo-β-*N*-acetylglucosaminidase or (EndoBI-1) found in *B. infantis* (Garrido et al., 2012). Large genomic islands found in *B. infantis* encoding transport and hydrolysis pathways for human milk glycans enable utilization of glycans released by EndoBI-1 from milk proteins (Garrido et al., 2011; Sela et al., 2008). In addition to HMOs, these *N*-glycans are thought to help facilitate colonization of the infant gut (Garrido et al., 2013).

Recent work showed that infants fed *B. longum* subsp. *infantis* EVC001 (*B. infantis* EVC001) were colonized at high levels by this organism and substantial changes to the infant gut microbiome and the metabolic output of this community were also observed. Among infants colonized by *B. infantis* EVC001, fecal concentrations of HMOs dropped significantly as *B. infantis* EVC001 fermented these oligosaccharides to primarily lactate and acetate. In contrast, neither the concentrations of fecal HMOs nor lactate and acetate changed in infants who had not been fed *B. infantis* EVC001 over the first month of life (Frese et al., 2017). We speculated that a homologous endo-β-*N*-acetylglucosaminidase found in *B. infantis* EVC001 may be active *in vivo* and be evidenced by the release of additional, selectively fermentable *N*-

* Corresponding author at: 2121 Second Street Suite B107, Davis, CA 95618, USA.
E-mail address: sfrese2@unl.edu (S. Frese).

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glycans (Karav et al., 2016) that may foster the growth of *B. infantis* EVC001 *in vivo*. In order to determine whether the endo-β-*N*-acetylglucosaminidase was active on human milk glycoproteins *in vivo*, we compared the *N*-glycan profile of fecal samples from breastfed infants colonized by *B. infantis* EVC001 and infants from the control group with different microbiome compositions lacking *B. infantis*. We hypothesized that the activity of an endo-β-*N*-acetylglucosaminidase (EndoBI-1) found in *B. infantis* could be detected by mass spectrometry targeting the unique *N*-glycans released from milk glycoproteins.

2. Materials and methods

2.1. Study design and subject characteristics

Fecal samples were collected from breastfed infants at Day 29 postpartum recruited in a previous study (Frese et al., 2017; Smilowitz et al., 2017). Samples were collected from two groups of infants and profiled by untargeted mass spectrometry (Karav et al., 2018) and is described in 2.2. One group was fed 1.8×10^{10} CFU per day of *Bifidobacterium longum* subsp. *infantis* EVC001 mixed with breast milk (EVC001-fed; $n = 8$), while the other group was fed breast milk alone (control; $n = 10$). Patient demographics are reported in detail previously (Karav et al., 2018) but overall, neither the subjects nor their mothers differed significantly ($P > 0.05$) across most demographic measurements. No difference was found between the groups in terms of among delivery mode (cesarean section or vaginally), duration of labor, rate of antibiotic administration to mothers during labor, complications associated with labor, gestational age at delivery, birth weight or length, sex, provision of antibiotics to infants, pre-pregnancy BMI, weight gain during pregnancy, group-B *Streptococcus* diagnosis, or maternal age. However, more infants in the control group were born to primiparous mothers ($P = 0.01$).

2.2. N-glycan data analysis

Spectra obtained from previously analyzed for colonic mucin-derived *O*-glycans were analyzed as described previously (Karav et al., 2018), except *N*-glycans were targeted using a library specific for *N*-glycans released by the activity of EndoBI-1. The overall approach was previously reported for these samples to determine the concentration of structurally similar HMOs by mass spectrometry (Frese et al), but instead, the analysis of spectra described here was specifically for *N*-glycans in this study. Fecal glycans were characterized on a nano-HPLC-Chip-TOF mass spectrometer using the methods previously described (Davis et al., 2016). As reported previously (Frese et al., 2017), the high-performance liquid chromatography (HPLC) system used was an Agilent 1200 series unit with a microfluidic chip, which was coupled to an Agilent 6220 series time of flight (TOF) mass spectrometer via chip cube interface. The sample was introduced into the TOF mass spectrometer via electrospray ionization, which was tuned and calibrated using a dual nebulizer electrospray source with calibrant ions ranging from m/z 118.086 to 2721.895, and data were collected in the positive mode (Davis et al., 2016). Untargeted mass spectra were collected and analyzed using Agilent MassHunter Work station Data Acquisition version B.02.01 on the nanoHPLC-chip/TOF. The "Find Compounds by Molecular Feature" function of the software was used to identify *N*-glycan structures potentially released from human milk glycoproteins by *B. infantis* EndoBI-1 (endo-β-*N*-acetylglucosaminidase). The software generated extracted compound chromatograms in the range of 400 to 3000 m/z, with an ion count cutoff of 600, allowed charge states of 1–3, retention times of 5–40 min, and a typical isotopic distribution of small biological molecules. The relative abundance (area) was calculated using MassHunter Profinder software. Example spectra are shown in Figure S1. To determine target *N*-glycan compositions, previously published libraries were used. However, these libraries were identified by previously validated tandem mass spectrometric analysis of glycans released using

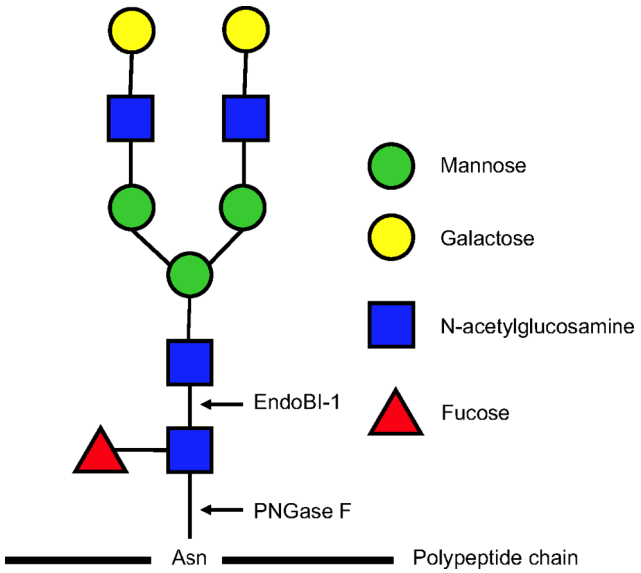

Fig. 1. PNGase F and EndoBI-1 are active on different locations relative to the *N*-glycan core, resulting in predictably altered *N*-glycan profiles.

PNGase F, which has a different activity on *N*-glycan structures. PNGase F cleaves the bond between the primary N-acetylhexosamine (HexNAc) and the polypeptide, whereas EndoBI-1 cleaves between the HexNAc bound to the polypeptide and the second HexNAc (Fig. 1). PNGase F activity is also limited when an alpha 1,3-fucose is attached to the primary HexNAc. Therefore, libraries obtained from previous studies were modified based on known differences between the enzymatic activities of PNGase F and EndoBI-1. Moreover, some free and conjugated glycans possess the same molecular mass although their chemical structures are different. Since tandem mass spectrometry was not performed in this study, these compounds, which could have been derived from either free or conjugated glycans were not considered during the data analysis and only unique *N*-glycan derived structures were examined. Compound abundances were expressed as volume in ion counts that corresponded to absolute abundances of the compounds in each sample. Numerical structural compositions are ordered as Hex-HexNAc-Fuc-NeuAc-NeuGc.

2.3. Microbiome sequencing

As previously described, DNA was extracted from samples for microbiome profiling. To assess how variations in bacterial communities in the infant gut were related to *N*-glycan profiles, 16S rDNA sequencing results reported previously from these samples (Frese et al., 2017; Karav et al., 2018) and deposited in the NCBI SRA (PRJNA390646) were compared to *N*-glycan profiles obtained as described above. Sequencing of 16S rDNA amplicons was performed at the UC Davis Genome Center on an Illumina MiSeq. This resulted in 9216 reads (SD ± 4505 reads) on average, per sample, after quality filtering (e.g. removal of low-quality reads or chimeric sequences). The relative abundance of the dominant bacterial families are shown in Table 3.

2.4. Statistical analysis

Wilcoxon Rank-sum test or Fisher's Exact test was used for single comparisons. *P* values, or FDR-adjusted *P* values, of 0.05 or less in comparisons were considered significantly different. Differences in bacterial community composition and *N*-glycans were calculated using principle coordinate analysis (PCoA) and a Bray-Curtis dissimilarity index between all *N*-glycan species was visualized via PCoA. To evaluate the effect-size of EVC001 colonization, both weighted UniFrac and Bray-Curtis dissimilarity matrices were tested via Permanova multivariate comparisons with 999 permutations and FDR-corrected *P*-

Table 1
N-glycan families detected in infant fecal samples. The mean (± SD) number of *N*-glycan isomers found in fecal samples of infants fed EVC001 and control subjects.

Number of *N*-glycans detected	Control (± SD)	EVC001-fed (± SD)	FDR-adjusted *P*-value
Total *N*-glycans	3.8 ± 2.82	42.37 ± 7.24	$P < 0.001$
Neutral Complex/Hybrid	3.1 ± 2.38	25.125 ± 3.14	$P < 0.001$
Acidic Complex/Hybrid (Sialylated)	0.1 ± 0.32	13.5 ± 4.24	$P < 0.001$
High Mannose	0.7 ± 0.95	3.25 ± 2.71	$P < 0.05$

values. *N*-glycan abundance was transformed to dissimilarity matrices using Euclidean distance while phylogenetic distance was obtained via the weighted UniFrac algorithm. Tests were performed using Pearson's product-moment correlation coefficient (*r*) with 999 permutations and a two-tailed test.

3. Results

3.1. N-glycan characterization

N-glycan compositions were determined by the untargeted approach of nano-HPLC-Chip-TOF. As human milk glycoprotein-derived *N*-glycans compositions were previously published, these libraries were used in this study (Nwosu et al., 2012). A library containing fifty-two *N*-glycan compositions was compared against the samples. Thirty structures (representing 49 total isomers) were detected. Infants fed EVC001 had a significantly higher number of distinct *N*-glycan structures (42.37 ± 7.24) than the control infants not colonized by EVC001 (3.8 ± 2.82) (Table 1). Among EVC001-colonized infants, an average of 25 ± 3.13 SD of them were neutral complex/hybrid, 13.5 ± - 4.24 SD were acidic complex/hybrid (sialylated) and 3.25 ± 2.71 SD were high mannose type of *N*-glycans. In contrast, control infants had fewer structures, primarily composed of neutral complex/hybrid *N*-glycans (3.1 ± 2.06) (Table 1).

3.2. Relative abundance of N-glycans

The abundance of each of the *N*-glycan compounds was determined by peak area (Table 2). Based on these findings, the relative abundance of samples from infants colonized with EVC001 and control samples were ranged between 163273 ± 204568 to 27509632 ± 24168794 and 0 to 1986832 ± 1994415, respectively. The most highly abundant compounds detected in EVC001 were 43000, 43100, 53000, 53100, 53200, 53010 and 53310. These compounds are different than a previously performed analysis based on PNGAse F by Dallas et al. (2011) which showed the highest abundant *N*-glycan compositions are 54210, 54110, 54200 and 54300. Differences observed were accounted by a single HexNAc given a difference in enzyme activity and substrate specificity between PNGase F, used by Dallas et al. (2011), and EndoBI-1.

The relative abundance of each class of *N*-glycans was determined for EVC001-fed infants and control samples are shown in Table 2. Based on the results, the total relative abundance of neutral complex/hybrid, acidic complex/hybrid (sialylated) and high mannose glycans of EVC001 fed samples were significantly different between the two groups (Table 2, Fig. 2).

These findings show that the majority of glycans detected in EVC001 fed samples are predicted to originate from lactoferrin and immunoglobulins (Fig. 3). When the compounds detected in our study were compared with the lactoferrin *N*-glycan library, 14 out of 18 *N*-glycans found in EVC001-fed infants could be attributed to lactoferrin but not immunoglobulins while a minority of these glycans were of nonspecific origin (i.e. they could originate from any one of multiple *N*-linked glycoproteins such as lactoperoxidase) or from milk immunoglobulins. These results suggest that *B. infantis* releases a substantial fraction of the *N*-glycans found on lactoferrin *in vivo*. High mannose, complex and hybrid glycans released from these glycoproteins were all detected.

3.3. N-glycan compositions are associated with B. infantis colonization

Given that there were significant differences in the abundance and diversity of *N*-glycans detected in stools from infants fed *B. infantis* EVC001 relative to the controls, the relationship between *N*-glycan abundance and diversity was examined in the context of the gut microbiome. We hypothesized that the difference observed between the two groups was a result of colonization with *B. infantis* EVC001. Overall, the gut microbiome composition is known to be predominantly *B. infantis* in infants fed *B. infantis* EVC001 (Table 3), however only weak associations between overall gut microbiome composition and *N*-glycan profile were observed for total *N*-glycans, when compared by Mantel test ($P > 0.05$). In contrast, the family *Bifidobacteriaceae* was significantly and strongly associated with total *N*-glycans, 41000, 43000, 43100, and 53000 (Table 4**)**. Together, these *N*-glycans alone composed 63.25% (± 19.51% SD) of the total *N*-glycan pool among fecal samples from infants fed EVC001; notably, in these samples the total glycan pool is also larger (7.92 Log_{10} intensity compared to 6.56 Log_{10} intensity in the control group, $P < 0.0001$). In comparison, only 16.21% (± 23.69%) of the total pool of *N*-glycans was composed of these four *Bifidobacteriaceae*-associated glycans in control infant fecal samples ($P = 0.0033$).

4. Discussion

While human milk provides nutrition for the infant, a substantial fraction of this food is not available as nutrients and serves a key function in shaping the gut microbiome (Zivkovic et al., 2011). Human milk oligosaccharides (HMOs) have been recognized for playing a major role in shaping the infant gut microbiome composition, but other glycan sources from food may play an additional role in the gut (Karav et al., 2016; Lee et al., 2014). *N*-glycans were recently shown to be released from milk glycoproteins by the activity of a unique, extracellular enzyme known as EndoBI-1, an endo-β-*N*-acetylglucosaminidase, found in the infant gut symbiont, *B. infantis* (Garrido et al., 2012; Karav et al., 2016). Further, the release of these *N*-glycans from milk proteins resulted in a freed glycan pool that was selective for *B. infantis,* even in comparison to other *Bifidobacterium* species, and remarkably, the released glycans were more effective at stimulating the growth of *B. infantis* once released from the protein or peptide, when compared to the intact protein itself or after the removal of glycans from the protein after pre-treatment with EndoBI-1 (Karav et al., 2016). This suggested that *N*-glycans released from glycoproteins *in vivo* may play an outsized role in shaping the microbial communities by supporting colonization of the infant gut by *B. infantis*. In agreement with previous *in vitro* studies, many of the *N*-glycans were significantly more abundant in fecal samples from infants colonized by *B. infantis* EVC001 compared to samples from control infants.

Glycan foraging is a noted competitive strategy for colonization by gut symbionts (Marcobal et al., 2013; Sonnenburg et al., 2005), though bound glycans such as *O*-linked glycans on mucin glycoproteins are less preferred by glycan foragers like *Bacteroides,* relative to larger, structurally repetitive plant starches and complex regulation of glycan consumption occurs through conserved pathways in some gut symbionts (Lynch and Sonnenburg, 2012; Marcobal et al., 2011). Unlike common strategies in these other glycan foragers where these oligosaccharides are degraded extracellularly to monomers, glycans released by EndoBI-1 under these conditions are released intact and transported by Family-1 Solute binding proteins (F1SBPs; Garrido et al., 2011; Sela et al., 2008) as a result of their conserved structural features shared with many HMOs.

Previous work *in vitro* demonstrated that EndoBI-1 releases of 52010, 53010, 44010, 53110, 43000 and 53000 from bovine milk glycoproteins and that these glycans are specifically utilized by *B.*

Table 2
Abundance (peak volume) of *N*-glycans found in EVC001 fed and control samples. Compositions are ordered by Hex-HexNAc-Fuc-NeuAc-NeuGc.

N-glycan type	*N*-glycan Code	Composition	*m/z*	z	m	Volume Control	Volume EVC001 Fed	FDR-adjusted *P* Value
Neutral Complex/Hybrid	1	41000	436.74	2	871.48	0 ± 0	984,402 ± 539,230	**0.0037**
	2	52000	618.23	2	1236.45	0 ± 0	727,585 ± 740,910	**0.0196**
	3	43000	639.74	2	1277.47	0 ± 0	16,336,429 ± 12,518,995	**0.0037**
	4	43100	712.76	2	1423.53	342,923 ± 731,341	11,473,565 ± 7,892,814	**0.0037**
	5	53000	720.77	2	1439.53	256,653 ± 541,647	27,509,632 ± 24168794	**0.0088**
	6	52200	764.33	2	1526.66	0 ± 0	170,903 ± 267358	0.0568
	7	53100	793.80	2	1585.59	925328 ± 1334063	4347701 ± 5648944	0.0986
	8	53200	866.83	2	1731.64	1,986,832 ± 1994415	5545213 ± 5803780	0.1290
	9	63100	874.33	2	1747.68	0 ± 0	311,166 ± 442663	0.0568
	10	35200	907.12	2	1825	0 ± 0	306844 ± 402042	**0.0260**
	11	45100	915.18	2	1829.49	0 ± 0	209082 ± 277573	**0.0260**
	12	45200	988.24	2	1975.4	0 ± 0	256041 ± 346230	**0.0260**
	13	36200	1008.81	2	2015.62	0 ± 0	687310 ± 832357	**0.0260**
	14	65100	1077.90	2	2153.79	0 ± 0	458324 ± 711077	**0.0260**
	15	36300	1081.77	2	2161.55	0 ± 0	154200 ± 173,652	**0.0260**
	16	52010	764.33	2	1526.66	0 ± 0	568612 ± 870952	**0.0196**
	17	64310	811.11	3	2534.2	0 ± 0	942611 ± 1506753	0.0568
	18	52110	837.83	2	1673.65	0 ± 0	163273 ± 204568	**0.0260**
	19	62010	845.65	2	1689.29	0 ± 0	406477 ± 797280	**0.0260**
Acidic Complex/Hybrid (Sialylated)	20	44010	886.82	2	1771.64	0 ± 0	613252 ± 1069722	0.0568
	21	53010	866.31	2	1731.62	17,234 ± 54499	2263998 ± 3337414	**0.0196**
	22	53110	939.34	2	1876.67	0 ± 0	788923 ± 1468401	0.1309
	23	44110	959.85	2	1917.70	0 ± 0	817721 ± 1201857	**0.0196**
	24	53210	1012.37	2	2023.73	20230.4 ± 63974	2374434 ± 3643257	**0.0260**
	25	53310	1085.90	2	2169.79	19,345 ± 61175	2594002 ± 3763059	**0.0260**
	26	51000	1034.37	1	1033.37	19,213 ± 60758	191175 ± 294378	0.1481
High Mannose	27	61000	598.21	2	1195.42	0 ± 0	400248 ± 573039	0.0568
	28	81000	760.09	2	1519.1	20,108 ± 63,589	207,358 ± 330,429	0.1796
	29	91000	841.13	2	1681.25	0 ± 0	433,937 ± 481,589	**0.0260**
Neutral Complex/Hybrid						3,511,738 ± 3,085,141	69,478,405 ± 48,954,788	< **0.001**
Acidic Complex/Hybrid (Sialylated)						56,810 ± 91,761	11,533,306 ± 8,831,971	< **0.001**
High Mannose						39,322 ± 82,925	1,232,719 ± 1,377,197	< **0.01**
All Glycans						3,607,870 ± 3,096,536	82,244,430 ± 55,275,913	< **0.001**

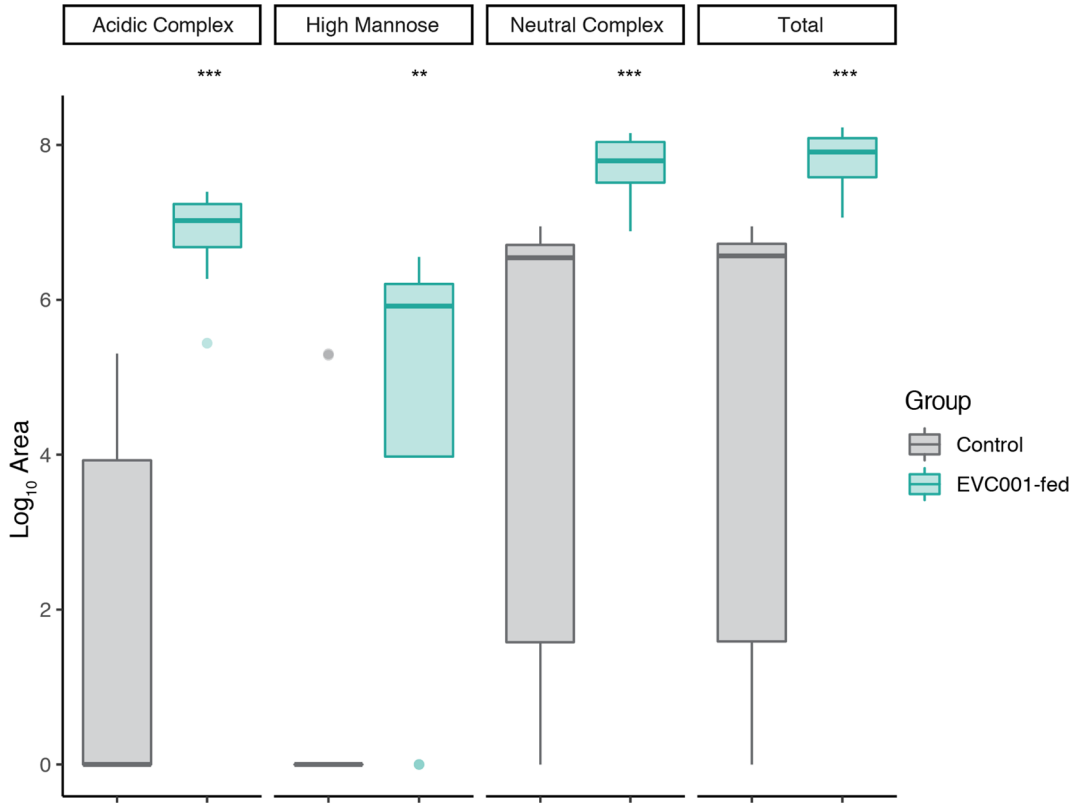

Fig. 2. Both the total area of all *N*-glycans and the combined area of all *N*-glycans as categories in three major subcategories (acidic complex/hybrid, neutral complex/hybrid, or high mannose) were significantly increased among infants fed EVC001 (teal boxplot), relative to the *N*-glycome found in control (grey boxplot) infants. ($P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***).

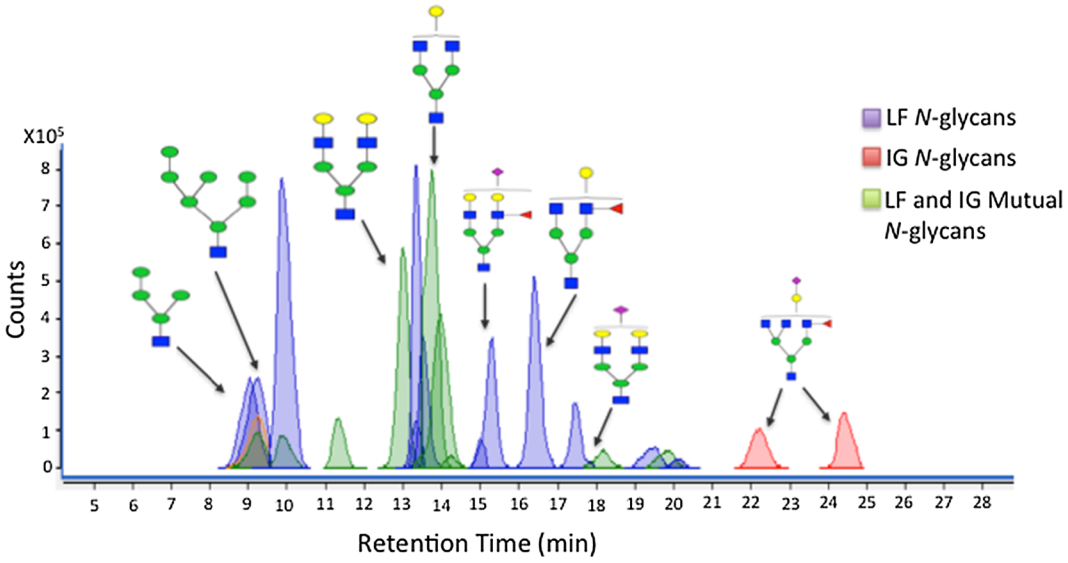

Fig. 3. Extracted compound chromatograms (ECC) for *N*-glycans predicted to be released from lactoferrin (LF), immunoglobulins (IG), or from shared *N*-glycans (including other milk glycoproteins). Purple, red and green peaks represent LF *N*-glycans, immunoglobulin *N*-glycans and mutual *N*-glycans for both glycoproteins, respectively. For the illustration of *N*-glycan structures, green circles, yellow circles, blue squares, red triangles and purple diamonds represent mannose, galactose, HexNAc, Fucose and NeuAc residues, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Mean relative abundance (percent, ± SD) of predominant bacterial families in infant fecal samples from both groups.

Bacterial family	Control	EVC001-fed
Bifidobacteriaceae	26.69 (24.23)	96.02 (2.69)
Bacteroidaceae	21.63 (14.28)	0.02 (0.05)
Enterobacteriaceae	19.02 (20.91)	0.77 (1.14)
Clostridiaceae	11.27 (17.15)	0 (0)
Lachnospiraceae	7.4 (18.2)	0.01 (0.01)
Streptococcaceae	2.24 (2.17)	1.91 (2.14)
Staphylococcaceae	1.49 (2.14)	0.4 (0.37)
Veillonellaceae	1.12 (2.68)	0.24 (0.6)
Enterococcaceae	0.52 (0.64)	0.19 (0.45)
Other	8.64 (7.4)	0.44 (0.49)

Table 4
Correlational analysis comparing microbiome composition (Mantel test) and significantly associated bacterial families (Spearman's ρ) with fecal *N*-glycans. Structural compositions are ordered as Hex-HexNAc-Fuc-NeuAc-NeuGc.

N-glycan structures	Mantel r	Mantel *P*-value	Bacterial family	Spearman's ρ	FDR-adjusted *P*-value
Total	0.11	0.19	*Bifidobacteriaceae*	0.70	0.02
			Enterobacteriaceae	−0.6	0.02
41000	0.23	0.05	*Bifidobacteriaceae*	0.78	< 0.001
			Enterobacteriaceae	−0.77	< 0.001
			Bacteroidaceae	−0.69	0.01
43000	0.09	0.38	*Bifidobacteriaceae*	0.82	< 0.001
			Enterobacteriaceae	−0.74	0.003
			Bacteroidaceae	−0.65	0.02
43100	0.11	0.33	*Bifidobacteriaceae*	0.72	0.01
53000	0.05	0.79	*Bifidobacteriaceae*	0.83	< 0.001
			Enterobacteriaceae	−0.82	< 0.001
			Lactobacillaceae	−0.63	0.03

infantis in vitro (Karav et al., 2016). Here we also detect all four of these glycans *in vivo* from exclusively breastfed infants and find that all but one (53110; Table 2) are significantly more abundant among fecal samples from infants fed EVC001, compared to samples from control infants. Interestingly, the mean fecal pH of infants colonized by *B. infantis* EVC001 is 5.1 (Frese et al., 2017; Henrick et al., 2018) and, together with the infant's approximate body temperature of 37 °C, these conditions mirror the previously determined optimum conditions for the enzyme, which enabled the release of these glycans from homologous bovine milk glycoproteins *in vitro* (Karav et al., 2015). Together, these results suggest that the enzyme is not only expressed *in vivo* but the conditions experienced *in vivo* as a result of colonization by *B. infantis* are similar to the enzyme's optimum previously determined empirically *in vitro*.

Under these conditions, the growth of *Enterobacteriaceae* in the infant gut is limited as a result of the canonical fermentation of carbohydrates to acetate and lactate by *Bifidobacterium* (Pokusaeva et al., 2011) thereby reducing fecal pH (Fukuda et al., 2011; Sorbara et al., 2019; Henrick et al., 2018). While we negatively correlated the abundance of *Enterobacteriaceae* and *Bacteroidaceae* with these glycans, it is unclear from these findings whether these structures directly affect the abundance of these taxa, or perhaps more likely, the abundance of these glycans is positively associated with *B. infantis* EVC001 colonization and contribute to the creation of conditions which disfavor the growth of Gram negative organisms. However, previous work has shown that *N*-glycans from milk proteins such as lactoferrin inhibit the adhesion of some enteric pathogens to gut epithelial cells and contribute to the antimicrobial activity of lactoferrin itself (Barboza et al., 2012; Karav, 2018). Further, the removal of *N*-glycans from proteins reduces resistance of the protein to protease degradation, resulting in the production of additional bioactive peptides from milk proteins (Garrido et al., 2013; Van Berkel et al., 1995) and which may limit the growth of potential enteropathogens.

5. Conclusion

Here, we show that an enzyme produced by a human gut symbiont, *B. infantis*, with unique function and structural specificity for the release of *N*-linked glycans found on milk glycoproteins is active under conditions encountered *in vivo*. The *N*-glycans produced by the activity of this enzyme are significantly more abundant and varied among infants fed *B. infantis* EVC001. Neutral complex/hybrid, acidic complex/hybrid, and high mannose glycans released by the activity of this enzyme are all significantly more abundant among infants fed *B. infantis*

EVC001. Further, these compounds were significantly correlated with the abundance of *Bifidobacterium*. Previous estimates suggest as much as 4–8% of the total mass of glycoproteins such as lactoferrin can be removed by the activity of EndoBI-1 as *N*-glycans (Karav et al., 2015; Karav et al., 2016), which, *in vivo,* could release as much as 800 mg of additional, highly-selective prebiotic glycans per ten grams of milk protein (either human or bovine) to the infant gut.

Ethics statement

The University of California Davis Institutional Review Board approved all aspects of the study (IRB #: 631099) regarding original study, sample collection, and analysis.

Declaration of Competing Interest

Steven Frese and Giorgio Casaburi are employees of Evolve Biosystems, Inc. Sercan Karav has received funding from Evolve Biosystems.

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Data availability

Raw sequencing data described in this study is publicly deposited in the NCBI SRA (PRJNA390646).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103485.

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