Chapter 8
Metabolomics and Milk: The Development of the Microbiota in Breastfed Infants

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Abstract Metabolomics provides a valuable strategy for describing and annotating the structures, compositions, and functions of mammalian milk. Detailed analyses of the complex components of milk have revealed an unexpected diversity of glycans consisting of oligosaccharides, glycoproteins, and glycolipids, all of which help shape the intestinal environment and in particular the intestinal microbiome of breastfed babies. Using complete and partial ensembles of glycan mixtures, the holistic principles of metabolomics analytics were leveraged for microbial screening studies. The complex glycans of human milk proved to be highly selective in their ability to support the growth of only a very rare group of enteric bacteria. These studies led to the conclusion that a signature achievement of breast milk is the development of a unique milk-oriented intestinal microbiota that results from a functional overlap of stereospecific glycan biosynthesis in maternal mammary epithelia with equally stereospecific glycosidase enzymes encoded within the genome of the commensal bacteria. Clinical evidence in support of that hypothesis has now been generated by the simultaneous administration and quantitation of the entire repertoire of glycans in the milk going in and the feces coming out of human infants. These platforms of systems biology combining separation technologies coupled to highly accurate and sensitive mass spectrometry with exhaustive library development and computational tools provide a model for success in understanding biological processes. Metabolomics is now extending that understanding of the infant

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microbiota and its phenotype to the role of complex glycans in the microbiota of all ages. The relentless selective pressure on the process of lactation within the mammary epithelial cell over millennia of evolution has been to nourish, protect, and support the survival of the mother infant pair. The principles that have emerged to nourish infants provide a guiding model for diet and health of all humans. The tools of metabolomics are proving successful in revealing the mechanisms behind milk's "genius."

Keywords Milk • Glycobiology • Microbiota • Infant • Oligosaccharides • Glycosidase • Glycan • Glycomics • Evolution • Lactation

8.1 Introduction

The mother and breastfed infant dyad provides a model to understand diet in its larger context. The scientific opportunities afforded by this model are transformative. Mechanistic insights to the targets of dietary inputs can be revealed by studying milk genomics, chemical composition, biological properties, and its diversity across mammals and temporally across lactation. The clinical comparison of exclusive breastfeeding against various formulas provides a powerful framework to study structurally defined diets and monitor the consequences of those structures on health and disease. In this respect, studies on milk and the lactating mammary gland provide unique insights into the mechanisms by which diet can act in protection and prevention. The mammary epithelial cell is a bioreactor for bioengineering complex structures and activities that act upon virtually all of the infant's processes: immunity, growth and development, metabolism, physiology, neurological development, and microbiota colonization and maturation. The components of milk execute on this biological blueprint using integrative and pleiotropic mechanisms that are difficult if not impossible to identify using the reductionist strategies of traditional biological chemistry. The comprehensive nature of the omic sciences and in particular metabolomics is changing the way milk is studied. Milk is the functional output of mammary metabolism, and it's a biofluid representing maternal genetics, health status, and environments.

A striking example of the principles and technologies of metabolomics applied to breast milk is in the area of glycomics; glycomics has revealed the diversity and abundance of glycans notably the free human milk oligosaccharides (HMOs) that are relatively unique to lactation and the glycosylated proteins, peptides, and lipids. Interestingly, glycans reach the large intestine and can ultimately be excreted and measured in the stool in healthy infants. This is a paradox if milk is considered a source of digestible nutrients for the infant. The resolution of this paradox is found in the fact that in most breastfed infants, these glycans disappear from stool, coincident with the appearance of a group of bacteria capable of digesting and utilizing them as growth substrates. The value of this relationship between maternal milk and

intestinal bacteria to shape infant postnatal development is still being revealed, ranging from protection from pathogenic bacteria, viruses, and toxins, to promoting neurological and immune systems and enhancing barrier function of intestinal epithelia. The highly selective digestibility of milk glycans act to shape the intestinal microbiome orchestrating its transition from the sterile uterus through the chaotic introduction of environmental bacteria at birth to a stable milk-oriented microbiome (MOM). This convergence of an entire metabolite class, glycans with the selective metabolism of bacteria and their interaction with the human host, is an opportunity to define metabolism as a dietary variable and study the structure-function relationships between diet, metabolism, and intestinal bacteria development. These studies provide broader principles for nourishing complex microbiota throughout life. At the core, comprehensive and accurate measurement of the structures and composition of milk's glycan metabolome is required.

Analytical chemistry has only recently brought the tools needed to measure glycobiology, the free oligosaccharides and glycans bound to proteins, peptides, and lipids in milk. Instrumentation is not sufficient; mass spectrometry must be coupled to separation technologies, enzyme biotechnologies, and bioinformatics tools to assemble all of the information into computationally accessible libraries. These technological advancements have led to the discovery that glycans are a central component of all mammalian milks, are variable across lactation and among women, and provide a wide diversity of structures to diverse functions [1–6].

8.2 Metabolomics and Human Milk

The simplifying elegance of the linear encoding of protein structure from DNA, RNA to protein sequence that is so empowering to biology from evolution to function is equally enabling to scientific research. Scientists have been wonderfully successful in annotating DNA-dependent biological processes because of the simplicity of linear sequence. Metabolism does not possess this simplicity. The dizzying complexity of metabolism must now be studied the old fashioned way, by measuring it. Scientists are beginning to assemble the technologies to measure metabolites in the accuracy, sensitivity, and comprehensiveness that reflect actual biology. A broad goal of food research is to build a linear understanding from the genetics of agricultural commodities, through their metabolism and thence composition as foods to the specific actions of those components on the metabolism and ultimately health of individual consumers (Fig. 8.1). Step one is to define the genetic and phenotypic basis of food composition through commodity growth and processing. The next step is to understand the principles by which human metabolism is controlled via these exogenous dietary components. This challenge will be particularly daunting in higher organisms due to the importance of structure to function. In higher organisms metabolites are distributed according to the cells, tissues, organs, and whole bodies. This structural dimension will demand that metabolites are measured as a function of the 3-dimensional structures of their immediate environment,

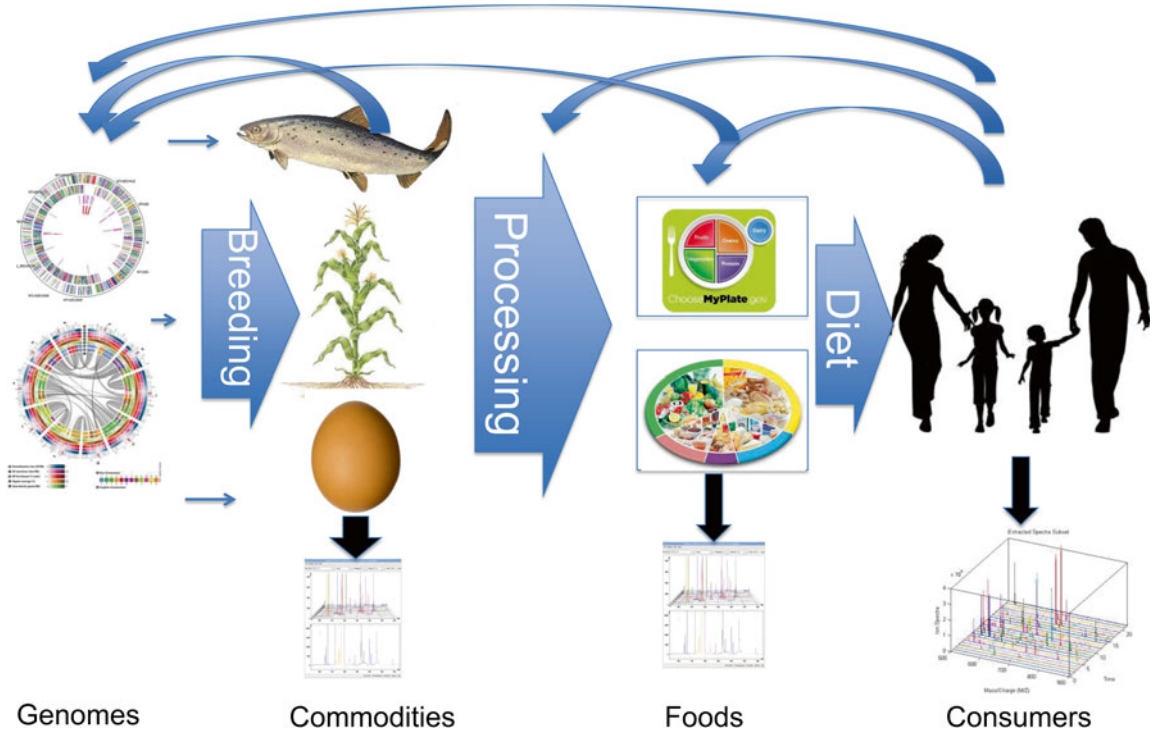

Fig. 8.1 The bidirectional flow of metabolic information through agriculture, food, and health. The genomics of agricultural commodities defines the metabolic machinery of farmed organisms which, once measured by metabolomics, can be guided by selective breeding and explicit genetic engineering. The metabolome compositions of harvested organisms that are the result of their metabolism are in turn alterable post-harvest by a wide variety of processing alternatives. The final compositions of chosen foods measured by food metabolomics define the overall diet compositions of individuals/families. Their diets influence their acute health which is in turn measureable by metabolomics of various body fluids. Departures from desired health trajectories detected by individual and population measurements then feed back into all of the agriculture and food input variables to alter diet composition and guide individuals to improved health

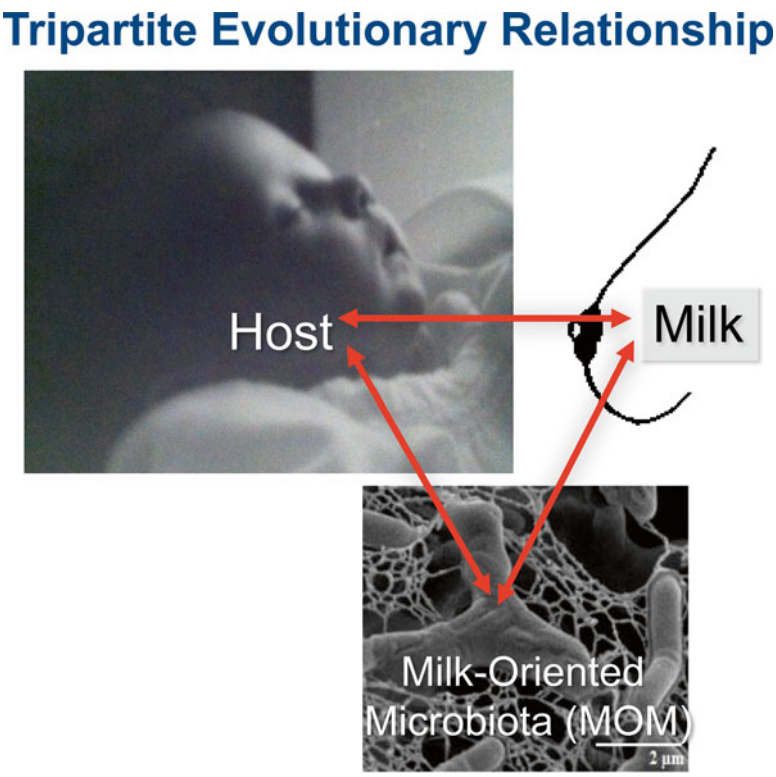

Fig. 8.2 The tripartite evolutionary relationship for mothers, infants, and their microbiota. The importance of the infant microbiota to its survival and genetic success is implied by the substantial investment of lactation in the control of this ecosystem. Understanding how and why lactation controls the infant microbiota provides scientific insights to microbial ecosystems in human intestine and beyond to all complex ecosystems in which food is a selective and discriminating input variable

techniques for which are only beginning to emerge. We have taken the approach of using the interaction between milk and microorganisms as a model for dietary metabolomics (Fig. 8.2). The principles emerging from this research provide scientists with examples of the interactions between diet and metabolism that may be instructive for higher animals.

8.3 Milk Glycomics

Glycans are the biopolymer class that has been largely ignored in spite of their abundance across the phylogenetic tree and through evolution [7]. Despite their importance in health and disease, they are not sequence encoded but rather the products of enzymatic metabolism. As a result of metabolic synthesis, the number of potential structures is massive contributing to the structural diversity seen in milk.

The complexity of a glycan is the result of a number of factors including branching, the number of different sugars, the stereospecific linkages of those sugars all leading to multiple isomers even for a single net mass to which must be added the dimension of conjugation: they are free or bound to proteins, peptides, or lipids again in a heterogeneous but stereospecific manner. Biology has apparently employed the combination of metabolism and structural diversity to leverage glycobiology into a variety of functions and most notably recognition. Perhaps not surprisingly, throughout evolution, the glycans on surfaces of cells are distinct both to "self" and to "foreign" organisms as the molecular basis for individuality. This dimension of diversity that is clearly of value to biological organisms is instead to scientists a nightmare precisely because there is no corresponding genetic template; every glycan must be explicitly analyzed to be identified. Research into the technologies and methodologies to routinely and comprehensively measure the glycans in biological and clinical samples is only now emerging, and as a result, a quantitative, metabolomics approach to glycobiology is becoming possible. Glycomics is defined as the systematic study of the total complement of sugars present in an organism in their free or protein and lipid-bound states [8, 9]. Researchers are now using glycomics to understand diet and health in the context of lactation, milk, and the role of complex glycans in various aspects of the development of the mammalian neonate.

8.3.1 Milk Oligosaccharides

The field of milk glycobiology is not new, and in fact many of the presumed roles of glycans throughout biology have been first discovered by examining milk. Nonetheless, the complexity of glycan structures has been a major hurdle to understanding specific structure – function relationships beyond binding assays. The oligosaccharides of human milk have become of particular interest in large part because they are an abundant (1–2 % w/v) and yet indigestible by the neonate. The biological challenges posed by this apparent paradox propelled a few key laboratories to pursue the analytical challenges of identifying and quantifying them. The revolution that they are bringing to analytical glycomics has been the result of innovations in separation science, enzyme biotechnology, mass spectrometry, automated library development, and computational toolsets.

8.3.2 Separation Science for Oligosaccharides

Liquid chromatography has been applied to glycan separation, yet neither normal nor reverse stationary phases provide sufficient separation power to successfully resolve glycan diversity in structure. Porous graphitized carbon (PGC) has been used as a uniquely selective stationary phase for bulk purification of glycans for

many years. Only recently has it been possible to formulate PGC as an HPLC stationary phase for the analysis of native oligosaccharides [10]. This combination of high-efficiency, high reproducibility, and stationary phase selectivity in an HPLC system has provided the first generation of separation platforms capable of the extensive separation of glycan isomers [11]. The future of oligosaccharide and glycan analyses will be extending this selectivity and efficiency to stereospecificity, quantitation, and throughput.

8.3.3 Stereospecificity of Glycan Structures

Glycans are nothing if not a forest of stereospecificity. The total number of possible structures imaginable is astronomical, yet because these glycan polymers are all the results of stereospecific enzymatic reactions, the actual number of structures found in biology is manageably finite and approachable by modern analytics and library systems. Nonetheless, precise oligosaccharide structures cannot be unequivocally identified on chiral separation phases and instead must still be determined the old fashioned way, by cleaving with stereospecific enzymes as an explicit step in the analysis [12]. The use of stereospecific enzymes will likely remain the most efficient means of assigning precise structures to oligosaccharides precisely because once a biological source is accurately described, it is not necessary to perform stereospecific analyses every subsequent analysis. The pragmatic proof of this principle has been demonstrated for the various milk oligosaccharides that have been analyzed for their stereospecificity [13].

The establishment of accurate metabolomics today cannot be achieved with online identification systems due to the complexity of the possible glycan structures relative to the separation platforms and mass spectrometry accuracy available. Instead effective methods for the structural identification of HMOs requires the construction of detailed libraries that map structures into analytical platforms taking advantage of the combinations of MS, tandem MS, and exoglycosidase digestion [12]. Neutral [14] and anionic milk oligosaccharides from humans [15] totaling 75 structural isomers have been annotated in this approach (Figs. 8.3, 8.4, and 8.5). Once begun, the library strategy has been extended to milks from other mammals to over 200 complete structures. This basic strategy is appropriate for the vast majority of applications to human milk biology since 50 structures represent 99 % of the total abundances of oligosaccharides in human milks [14–16].

Metabolomics is of relatively little utility if it only identifies structures and cannot quantify the absolute amounts of metabolites within biological samples. As a subset of the metabolome, glycan quantitation remains a major obstacle to metabolomics of glycobiology. Oligosaccharides lack discriminating chromophores for spectral detectors. As a result, oligosaccharides are often derivatized with absorbing labels including anthranilic acid (AA) or 2-aminobenzamide (AB) for quantitation [17]. To date the varying ionization efficiencies of glycans compromise the use of mass spectrometry of oligosaccharides. The alternative, using isotopically

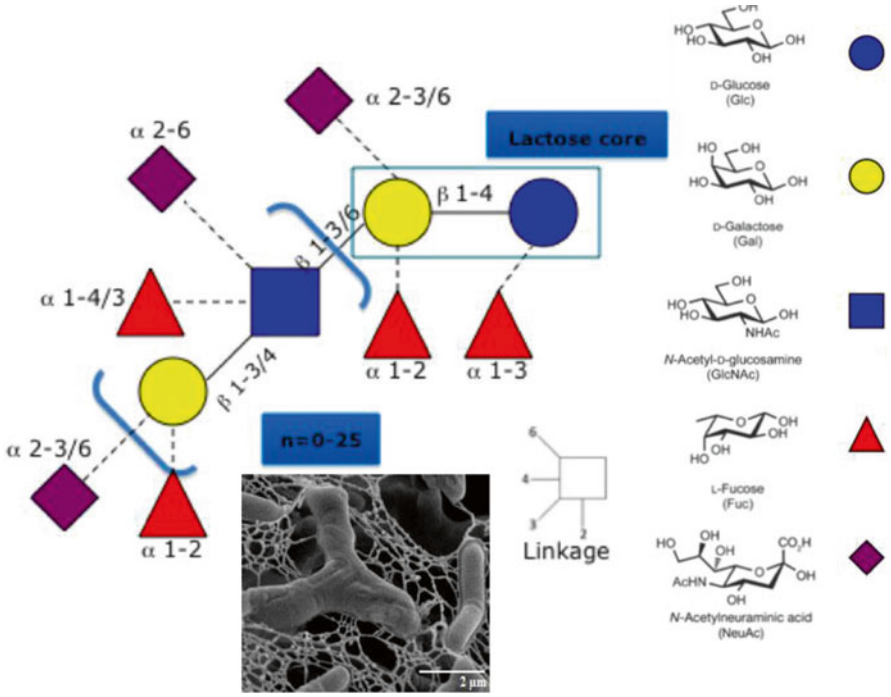

Fig. 8.3 Basic oligosaccharide structures in milk. The structural core of oligosaccharides is illustrated, the key sugar monomers that make up the oligosaccharide compositions and the possible stereospecific glycosidic bonds that are possible. *Bifidobacteria longum* subspecies *infantis* contains the genetic capability to synthesize ostensibly all of the enzymes necessary to cleave this array of complex glycans

enriched internal standards, while making MS highly accurate [18], requires the synthesis of the entire library of potential structures which is not currently available. Quantitation of metabolites remains the great challenge for the applications of metabolomics to its most relevant applications in health.

The presence of oligosaccharides has been confirmed in very early mammals and marsupials [19]. Thus, indigestible carbohydrate biopolymers provided a selective advantage throughout mammalian lactation. This advantage has apparently continued up to humans. Human milk contains greater concentration and diversity of soluble oligosaccharides than other mammalian milks [20] ranging from on average 7 g/L mature to 23 g/L in colostrum [21, 22]. These soluble oligosaccharides are composed of glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid (NeuAc) monosaccharides. The basic biochemistry of oligosaccharide synthesis in the mammary gland is initiated by a lactose core of Gal and Glc catalyzed by β-galactotransferase in the presence of α-lactalbumin. The vast majority of HMO structures are based on this lactose core [23]. Lactose is then decorated by β1–3 linkage to lacto-N-biose (GlcNAc linked to Gal by β1–3 linkage) or by β1–6 linkage to N-acetyllactosamine (GlcNAc linked to Gal by β1–4 linkage).

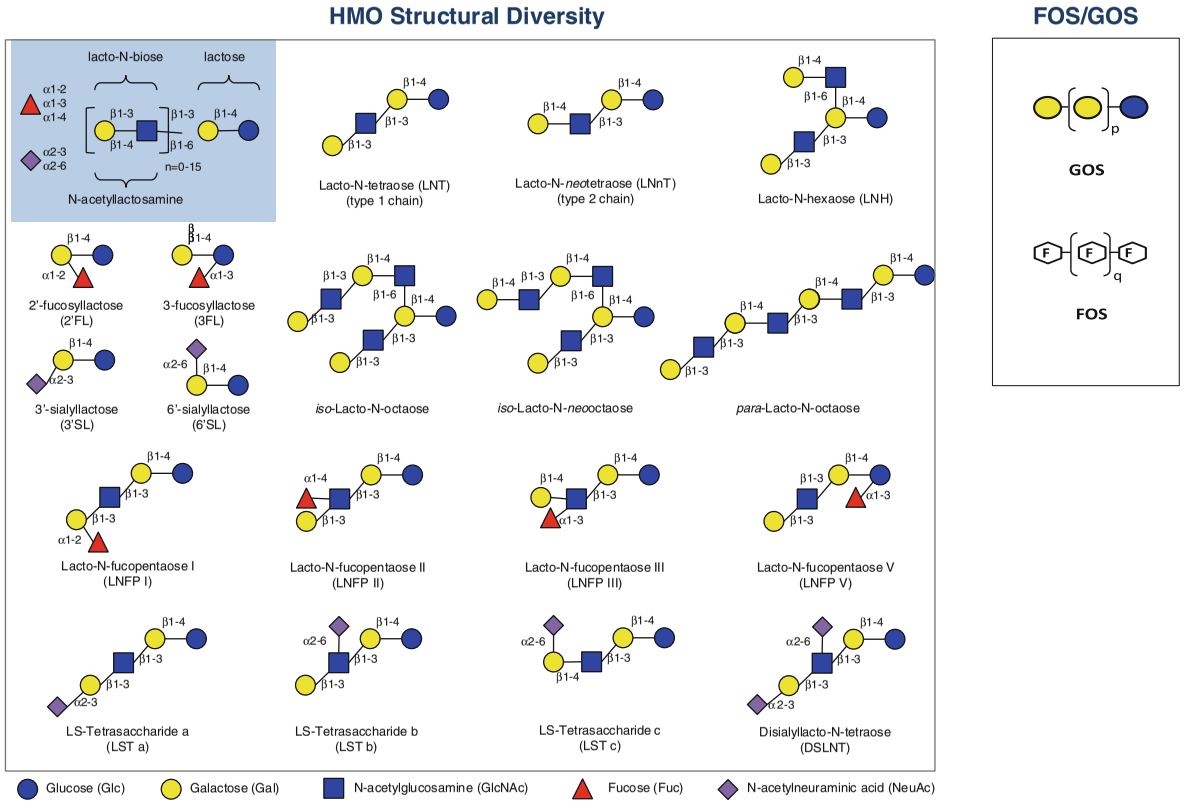

Fig. 8.4 Structural diversity of complex free glycans in human milk. The cartoon approximations of a subset of oligosaccharide structures in human milk attest to the diversity and yet specificity of this biomolecule class. Milk oligosaccharides as highly structured biomolecules are the result of multiple sugars joined by multiple stereospecific linkages

					Composition				Abundance (counts per second)		
	HMO	Structure	Mass	RT	Hex	Fuc	HexNAc	NeuAc	Milk	Feces	Urine
1	3'FL		490.190	1.31	2	1	0	0	NO	NO	NO
2	2'FL		490.190	11.69	2	1	0	0	NO	NO	NO
3	3'SL		635.227	22.330	2	0	0	1	58916	191249	370266
4	6'SL		635.227	15.231	2	0	0	1	30524	50386	NO
5	LOFT		636.248	14.470	2	2	0	0	NO	11395	11197
6	6'SLN		676.254	14.998	1	0	1	1	NO	59563	115044
7	3'SLN		676.254	23.061	1	0	1	1	NO	NO	227705
8	LNT		709.264	15.090	3	0	1	0	21650694	11349391	4096506
9	LNOT		709.264	15.200	3	0	1	0	410714	772639	83004
10	3'Sle		822.312	14.609	1	1	1	1	NO	NO	4368

Fig. 8.5 Extracted page of the table from the entire library of oligosaccharides in milks. Table illustrates the data possible to acquire with modern glycomics platforms including accurate mass, retention time, sugar subunits, and the abundance in milk and in various biofluids from infants

This growing chain structure can be further elongated with lacto-N-biose and N-acetyllactosamine by β1–3 and β1–6 linkages; Fuc connected with α1–2, α1–3, or α1–4 linkages and/or NeuAc residues attached by α2–3 or α2–6 linkages at the terminal positions (Fig. 8.3). The terminal sugars are particularly diagnostic of different mammalian milks, 60–80 % of HMOs are fucosylated, and 10–15 % of HMOs are sialylated in human milk [24].

8.4 Annotating the Functions of Human Milk Glycans

The structures of milk oligosaccharides have been selected for an unusual biological value: not to be consumed by infants. This selective pressure on lactation has been particularly intense since milk is the sole source of nourishment for mammalian infants. The genetics, synthesis, and structures of oligosaccharides in milk are unequivocally discoverable. However, the functions of oligosaccharides that were the basis for their emergence and persistence through evolution are not as easily discovered. The process of understanding their actions must first identify their actions, and then each of these actions must then be tested mechanistically as an actual valuable function in vivo. The most extensive approach to evaluating the actions and potential functions of oligosaccharides in human milk has been to establish the detailed support of the growth of specific strains of bacteria notably bifidobacteria [25, 26]. While the mechanisms and extent of microbial diversity in breastfed infants are still being actively documented, the basic observation that bifidobacterial species dominate the microbiota of breastfed infants around the world compared with formula-fed infants has been well established [27]. How an intestinal microbial ecosystem maintains a dominant and consistent bacterial population in the face of repeated and diverse inoculations with environmental microorganisms has been largely speculative until recently. Research has revealed the remarkable interaction between the stereospecific linkages defining the structures of milk oligosaccharides and the genetic repertoire of stereospecific glycosidases and solute-binding proteins that provide these bacteria a distinct competitive growth advantage within the intestine of the breastfed infant.

8.4.1 Screening Bacteria for Growth on Oligosaccharides

In an ongoing search for biological activities of these molecules to justify their abundance and diversity in milk, a prevailing hypothesis was that they are substrates for bacterial growth. However, no studies had yet documented that fact nor whether growth was selective among bacteria. Initial growth experiments in fact failed to demonstrate significant growth of bacteria when human milk oligosaccharides were the sole source of carbon in an otherwise supportive medium [25]. A series of subsequent experiments revealed that among gut-related bacteria tested (including *Lactobacillus*, *Clostridium*, *Eubacterium E. coli*, *Veillonella*, *Enterococcus* isolates), only *Bifidobacterium* and *Bacteriodes* species grew to high cell densities [28]. Growth on HMO was found in a select group of *B. bifidum* and *B. longum* subsp. *infantis* strains. In these same isolated growth conditions, even isolates of *B. longum* subsp. *longum* and *B. breve* showed poor growth, and other strains of *B. adolescentis* and *B. animales* were ostensibly unable to grow on HMO [29] (Fig. 8.6).

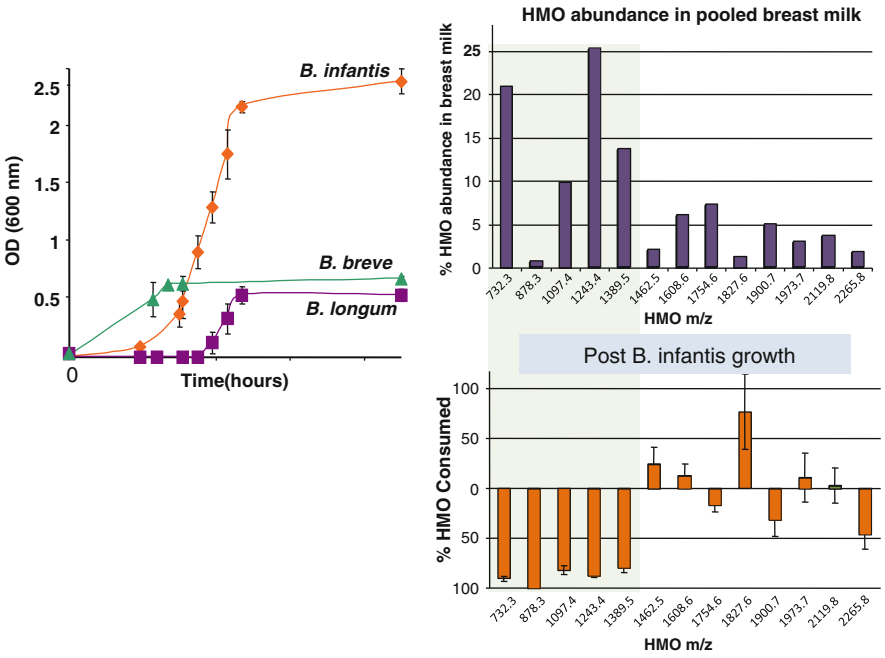

Fig. 8.6 Growth curves and human milk oligosaccharide consumption by *Bifidobacteria longum* subspecies *infantis*. The growth curves of isolated bacteria (indicated) on media containing only isolated milk oligosaccharides as carbon source of *B. longum*, *B. longum* subspecies *infantis*, and *B. breve* are shown, illustrating the conspicuously greater growth of *B. infantis*. The most abundant oligosaccharides in human milk are histogrammed in the figure on the *right* before and after consumption by *B. infantis* illustrating the complete consumption of the majority of oligosaccharides by this bacterium [25]

The complex mechanisms by which milk oligosaccharides guide bacterial growth within the ecosystem of the infant intestine have been elaborated in a series of microbial studies. Among the bifidobacteria that are able to consume HMO, different strategies are present to use HMO as a substrate. In isolated growth studies of HMO consumption, *B. longum* subsp. *infantis* ATCC15697 most efficiently consumed oligosaccharides seven sugars (DP) or below [25]. Oligosaccharides below ten sugars are the majority of species human milk [10]. Other bifidobacteria including *B. longum* subsp. *longum* DJO10A and *B. breve* ATCC15700 that grew slowly on pooled HMO were found to be consuming mostly a single, nonfucosylated/nonsialylated species, LNnT. LNnT is present in breast milk yet a small portion of the overall HMOs. *B. breve* did grow in culture on all the monomer constituents of HMO and thus if present within the gastrointestinal tract could grow on liberated monosaccharides.

The bacteria that were found to be capable of growing on HMO were analyzed for the presence of metabolic activities towards complex oligosaccharides including the key sialidase and fucosidase activities required to deconstruct complex glycan

structures. Among the strains examined, fucosidase activity was present in *B. longum* subsp. *infantis* and was only detected upon growth on HMO [25].

Distinct strategies for catalytic activity on complex biopolymer destruction are known among intestinal bacteria. The majority of intestinal bacteria secrete extracellular glycosidase enzymes that liberate free sugars that are then subsequently taken up by bacteria and metabolized. Select bifidobacteria use lacto-N-biosidase activity to break down oligosaccharides [30]. LNB is transported into *B. bifidum* via an ABC transporter and an associated LNB-specific solute-binding lipoprotein whereby it is further processed and fed into the central metabolic pathway [31].

The discovery that *B. longum* subsp. *infantis* ATCC15697 was uniquely capable of growing on human milk oligosaccharides led to an immediate project to sequence its genome. No prior experience prepared the investigators for the elegance of the genetic repertoire of this organism's sequence. *B. longum* subsp. *infantis* ATCC15697 has become the blueprint for understanding the genetic basis of glycan-specific growth and phenotype [32]. This specific strain possesses clusters of genes associated with its unique phenotype distributed in the genome into four loci. The most informative, HMO cluster 1 (Fig. 8.7), contains all the necessary glycosidases (sialidase, fucosidase, galactosidase, and hexosaminidase) and transporters necessary for importing and metabolizing HMO. Sequencing more isolates for HMO-related genomic architecture among *B. longum* subsp. *infantis* isolates provides a detailed genetic map of the mechanisms behind the vigorous growth of this clade on HMO.

The bacterial model of metabolomics illustrates the complexity of structure within metabolic pathways. Within the large HMO cluster (Fig. 8.7) are genes encoding an interesting group of extracellular solute-binding proteins (SBP; pfam 01547) demonstrated to bind oligosaccharides. These proteins provide two functions for the bacteria in their ecological niche of the breastfed infant intestine. These solute-binding proteins would tether the bacteria to glycans on the luminal side of the infant intestine and provide a net coverage of microbial binding sites thus blocking potential pathogens from the infant. Of more direct value to the bacterium, these solute-binding proteins would internalize free oligosaccharides directly infusing substrate into its endogenous metabolism. This substrate sequestering mechanism provides the *B. longum* subsp. *infantis* a unique foraging advantage in the overall microbial community. These solute-binding proteins also appear to be of singular advantage to the mammalian infant gut. A subset of these genes shows a pronounced evolutionary divergence from other SBP family 1 proteins in bifidobacteria [32]. The emergence of these genes is consistent with their functions as a mechanistic basis of symbiosis with humans through their interaction with milk oligosaccharides. The *B. longum* subsp. *infantis* genome has been shown to contain 21 family 1 SBP, more than most bifidobacteria.

The results of genomic analyses of bifidobacteria illustrate that HMO-related clusters are shared among all *B. longum* subsp. *infantis* isolates that have been examined to date, yet they are notably absent in other sequenced bifidobacteria, such as *B. longum* subsp. *longum* DJO10A [33] and *B. adolescentis* ATCC15703

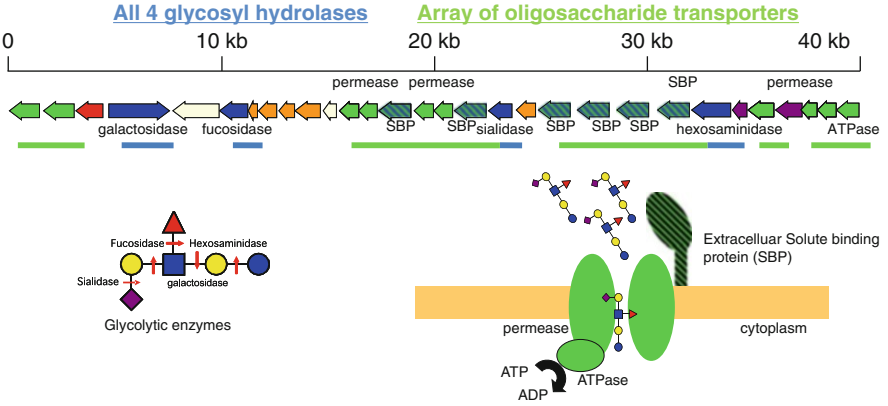

Fig. 8.7 Gene cluster 4 from the complete genome of *B. infantis* illustrating the location of the genes encoding glycosidases and oligosaccharide transporters [32]. The putative glycosidic linkages on which the glycosidic enzymes react are shown below left and the model of the cell membrane-bound solute-binding protein complex is shown *below right*

(GenBank AP009256), which grow weakly or not all (respectively) on HMO [29]. The elegance of microbial genetics is illustrated by the HMO-related gene set shared between ATCC15697 and DJO10A. This seven-gene operon is responsible for LNB metabolism further evidence of evolution selecting for metabolic substrate utilization [34]. Given that DJO10A is able to weakly grow on HMO and glycoprofiling indicated a small consumption of LNnT, it is tempting to speculate that this operon is linked to consumption of that particular HMO moiety.

While it is very hard to generalize the mechanisms of HMO catabolism across bifidobacteria because of strain heterogeneity and taxonomic confusion [35] within the genera, several important trends have emerged. The most common infant-borne bifidobacteria, *B. bifidum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, and *B. breve*, possess different modes for consumption of HMO (Fig. 8.6). *B. longum* subsp. *infantis* likely imports the lower molecular weight oligosaccharides via an army of dedicated ABC transporters. Once inside the cell, these oligosaccharides are catabolized by a complement of glycosidases prior to entry of the monosaccharides into central metabolic pathways. In contrast, *B. bifidum* exports fucosidases and lacto-N-biosidase to remove LNB from the HMO structure (leaving the free fucose and sialic acid behind) [48], internalize the free LNB and catabolize it intracellularly. Both *B. breve* and *B. longum* subsp. *longum* are able to consume free LNnT from an HMO pool, whereas *B. breve* can also grow on the various monomer constituents of HMO [48]. These different strategies suggest a possible mechanism for niche partitioning among the different bifidobacterial species within the developing infant gastrointestinal tract microbiota. Taken together this data provides a mechanism of action for glycan structures.

8.4.2 *Prebiotics for Infant Gut Bifidobacteria*

Breastfed infants that are colonized by protective strains of bifidobacteria benefit from the microbial activities within their developing intestine [36], which supports a valuable function in vivo for the emergence and persistence of glycans. Henry Tissier observed by microscopic analysis and culture techniques that the feces of breastfed infants were unique in containing a bacterial isolate he termed "*Bacillus bifidus communis*" [37]. For the 100 years since that initial identification, methodological techniques have wrestled to accurately type much less understand the specific bacteria within breastfed infants largely due to technical problems [38–40]. The challenges have become understandable in retrospect. Initial, culture-based studies failed to isolate significant proportions of bifidobacteria from infants, but these culture techniques failed to appreciate the oxygen sensitivity of infant bifidobacteria and were omitted. The major breakthroughs in DNA-based culture-independent methods should have identified bifidobacteria, yet unfortunately the 16s rDNA primers that are the basis of detection in these methods were not designed to effectively amplify bifidobacteria. Finally, both 16s rDNA surveys and metagenomic techniques that ostensibly sequence all DNA and again should have unequivocably identified bifidobacteria failed to appreciate the physical integrity of the double cell wall of bifidobacteria and the need to selectively handle the disruption of these barriers to DNA release for sequencing. These technical difficulties are now being resolved, and accurate measures of infant fecal microbiota are now available. With these techniques in place, studies are demonstrating very high proportions of specific strains of bifidobacteria in breastfed infants prior to transition to an adult microbiota [41]. The analyses of the breastfed intestinal track have revealed *Bifidobacterium longum* and *B. breve* with *B. bifidum* and *B. pseudocatenulatum* and *B. catenulatum* also present [42].

The basic concept that milk itself was influencing the microbial population was proposed by Gorgy and colleagues [43] on the basis of observations that *B. bifidum* (then termed *Lactobacillus bifidus*) grew on human milk fractions. The concept however implied that there was a single component responsible, the so-called *Bifidus* factor. Various studies since have demonstrated that human milk does indeed contain indigestible matter that since humans cannot break them down into digestible monomers would invariably reach the intestine [44–46]. The selectivity of growth promotion by bifidobacterial species growing on human milk oligosaccharides was first demonstrated in vitro by Ward et al. [47, 48]. A series of detailed studies have extended this initial observation demonstrating that only certain bifidobacterial species consume the majority of the stereospecific oligosaccharides of human milk [1, 25, 42]. Within specific strains, growth on oligosaccharides differed leading to the conclusion that *B. infantis* and select *B. breve* preferentially consume fucosylated and sialylated HMOs. These results indicate that bifidobacterial strains that grow well on specific glycan structures possess genetic adaptations for select growth on human milk in the infant intestine [32, 49].

The interaction between human milk oligosaccharides and bifidobacteria provides a unique opportunity to map the continuum of metabolites from a food, through the genetics of their disassembly by a "consumer" through the metabolic pathways that utilize them for growth. The genes in bifidobacteria that specifically bind and catabolize HMOs for energy have been identified, expressed, and verified for enzymatic activity [25, 28, 50–53]. The process of annotating these genes has demonstrated that different bifidobacterial species grow on HMO by distinct catalytic mechanisms. *B. infantis* possess a 43-kb gene cluster (termed HMO cluster I), encode for glycosyl hydrolases, and transport systems using a unique and highly efficient pathway to internalize and metabolize milk oligosaccharides [54, 55]. In contrast, *B. bifidum* is equipped with genes encoding a different set of catalytic activities toward HMO consumption. This strain exports fucosidases and a lacto-N-biosidase to hydrolyze lacto-N-biose from HMO structures which is in turn transported into the bacterium and metabolized [56].

The process of annotating the detailed mechanisms of the metabolism of human milk oligosaccharides by bifidobacteria has revealed consequences of that metabolism that were unanticipated. This group of metabolites causes a fundamental shift in the phenotype of the bacterium itself. Milk oligosaccharides trigger a specific HMO phenotype to *B. infantis*. In effect the bacterium shifts to a phenotypic state that is linked to its competitive success in establishing itself within the microbial ecosystem. The phenotype is also associated with interactions between the bacterium and the infant host. Chichlowski et al. [57] reported that the HMO-specific phenotype of *B. infantis* ATCC15697 on HMOs increases binding to intestinal epithelial cells in vitro. These studies suggest that the specific phenotype of bifidobacterial populations grown on human milk oligosaccharides as metabolites provides mechanisms to the organism supporting greater growth, microbiota persistence interactions with the host epithelium. This model of a metabolically distinct bacterial population induced by its "food" source is supported by in vivo administration of *B. infantis* to premature infants fed either formula or breast milk. The human breast milk-fed infants, when supplemented with *B. infantis*, had increases in fecal bifidobacteria and decreases in *γ-Proteobacteria* compared with the formula-fed group [58]. The ability of these specific bacteria to deconstruct HMOs that is encoded in their genome suggests the co-evolution of human lactation and specific commensal organisms. Thus, mothers are shaping the protective milk-oriented microbiota (MOM) of their infants through breast milk (Fig. 8.8) [53, 59]. This is one example of how milk glycans are being annotated.

8.5 A Vision for Metabolomics in the Future

The science of nutrition is faced with a daunting challenge: improving human health. The enabling principles of reductionist chemistry that were so effective in identifying essential nutrients are failing to address the more complex problems of non-communicative but diet-dependent diseases that are epidemic around the world.

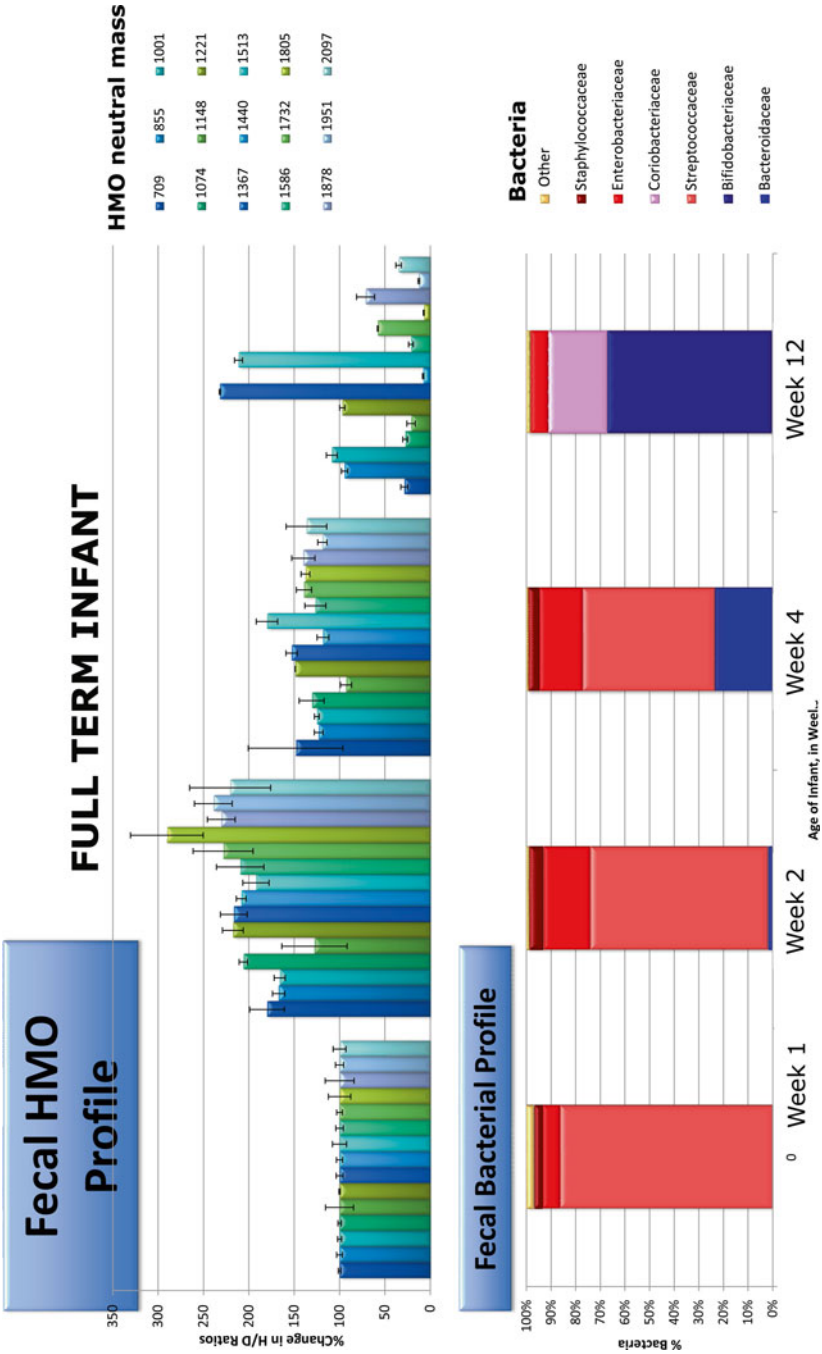

Fig. 8.8 Fecal analyses of a single infant at several time points through the first 3 months of life. *Above* are shown the oligosaccharides from human milk in the infant feces, normalized to week 1 in which all oligosaccharides from milk appeared unmodified in the feces. Successive weeks later time points illustrate the oligosaccharide abundances in the feces relative to week 1. *Below* is shown the bacteria profile of the same fecal samples through the first 3 months of life. (modified from de Leoz [60] unpublished)

Single molecules delivered to everyone in the population will not solve these problems. Nutrition as a field must lead the world into more integrative, biology-driven strategies that are not only quantitatively precise and mechanistically complete but mapped to actual foods and deployable as individual solutions. The first proofs of principle of such strategies are emerging from the, admittedly more narrowly defined, nourishment of breastfed infants. The toolsets of systems biology including genomics, metabolomics, proteomics, and glycomics have shown their power to interrogate the complexity of milk and reveal how it accomplishes an astonishingly successful biological feat, the colonization and development of the infant microbiota. Evolution clearly identified this to be an important target for mammalian health. Human mothers are nourishing the bacteria within their infants almost as enthusiastically as their infants. Yet, the strategy of nourishing the infant microbiota is a lesson for all of nutrition research. Rather than a single, simple molecule, the mammary gland produces an entire metabolome that includes: a spectrum of complex oligosaccharides and glycans that evade digestion by the infant and continue through to the infant's lower intestine. The complexity of glycans provides an intense selectivity that rewards only those bacteria genetically capable digesting the glycans and accessing their sugars. The combination of glycan complexity as available substrate and genetic capability as enzymatic specificity is a model for nutrition's microbiota research going forward. The knowledge assembled to date has begun the process of mapping the detailed mechanistic understanding of the functions of different microbial ecosystems in the infant. Key questions remain: How does a particular microbiota protect infants from pathogens and what are its weaknesses? How does a particular microbiota educate immunity in the face of the bewildering array of both pathogenic insults and completely benign passersby, and what are the causes of its failures? How does the microbiota prevent the massive activation of immunity and the anticipated increase in inflammation that would be expected from dropping a naïve, ostensibly sterile infant in the "real world," and can we apply these same principles to adults? How does a particular influence whole body metabolism and ensure appropriate food intake and suitable direction of fuels to peripheral tissues, and could these same mechanisms take visceral fat out of adults and put back "baby fat"? The successes of the first generation of metabolomics research tools applied to understand the interactions between mammary-produced oligosaccharides, and the infant microbiota are a glimpse of what this new field of biology can achieve.

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