Evolution of milk oligosaccharides: origin and selectivity of the ratio of milk oligosaccharides to lactose among mammals

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Abstract

Background: The carbohydrate fraction of mammalian milk is constituted of lactose and oligosaccharides, most of which contain a lactose unit at their reducing ends. Although lactose is the predominant saccharide in the milk of most eutherians, oligosaccharides significantly predominate over lactose in the milk of monotremes and marsupials.
Scope of review: This review describes the most likely process by which lactose and milk oligosaccharides were acquired during the evolution of mammals and the mechanisms by which these saccharides are digested and absorbed by the suckling neonates.
Major conclusions: During the evolution of mammals, c-type lysozyme evolved to α-lactalbumin.This permitted the biosynthesis of lactose by modulating the substrate specificity of β4galactosyltransferase 1, thus enabling the concomitant biosynthesis of milk oligosaccharides through the activities of several glycosyltransferases using lactose as an acceptor. In most eutherian mammals the digestion of lactose to glucose and galactose is achieved through the action of intestinal lactase (β-galactosidase), which is located within the small intestinal brush border. This enzyme, however, is absent in neonatal monotremes and macropod marsupials. It has therefore been proposed that in these species the absorption of milk oligosaccharides is achieved by pinocytosis or endocytosis, after which digestion occurs through the actions of several lysosomal acid glycosidases. This process would enable the milk oligosaccharides of monotremes and marsupials to be utilized as a significant energy source for the suckling neonates.
General significance: The evolution and significance of milk oligosaccharides is discussed in relation to the evolution of mammals.

Key words; milk oligosaccharides, evolution, monotremes, marsupials, eutherians, α-lactalbumin, human milk oligosaccharides, bifidobacteria

1. Introduction

Mammalian milk and colostrum usually contain lactose as the predominant saccharide among a variety of oligosaccharides, each of which has a lactose unit at its reducing end. Human milk contains 12-13 g/L of oligosaccharides, which constitute the third largest solid component in milk after lactose (60 g/L) and lipids (35 g/L). Human milk oligosaccharides (HMOs) are thus one of the major components of breast milk. To date 257 varieties of HMOs have been isolated, of which 169 have been structurally characterized. It has been shown that HMOs have biological significance as prebiotics which stimulate the growth of beneficial colonic bacteria, as anti-infection agents that inhibit the attachment of pathogenic microorganisms to the colonic mucosa and as immune modulators that reduce inflammation. In breast-fed infants they also act as agents which protect against necrotizing enterocolitis, as factors that strengthen the colonic barrier function, and as brain-nerve stimulating factors. The structures and biological functions of HMOs and of bovine and caprine milk oligosaccharides have been reviewed in several publications. In this review, we describe a hypothesis concerning the evolution of milk oligosaccharides in relation to mammalian evolution, as well as the history of studies on milk oligosaccharides in a variety of species. Although we have previously reviewed these topics [1-5], in this paper we attempt to improve our hypothesis, based on additional data described in previous publications.

2. Development of lactation in mammary glands during mammalian evolution

Vertebrates comprise fish, amphibians, reptiles, birds and mammals. The definitive feature of female mammals is the secretion of milk. Living mammals are grouped into three subclasses: monotremes, marsupials and eutherians. Recent evidence suggests that monotremes (platypus and echidna) diverged from the common ancestor of mammals about 187 million years before present (MYBP) [6]. Marsupials and eutherians diverged at least 135 MYBP [7].

The three mammalian subclasses have very different reproductive strategies. A unique characteristic of monotremes is their oviparous mode of reproduction; their young are hatched from eggs instead of emerging from a uterus. This must be a character that originated from a common ancestor. Monotremes have a number of other ancestral features not shared by other mammals, such as nipple-less lactation, cervical ribs and dwarf nephrons. Marsupials, like monotremes, have an extremely short gestation period compared with eutherians of similar size; the weights of newborns are 10 mg to 2 g depending on the marsupial species, indicating that they are very immature and undeveloped. Marsupial

newborns exhibit only a few developed tissues, such as those in the forefeet and in the olfactory system that enable them to reach the pouch of the mother, and mouths that work to suck milk from a nipple in the pouch, to which they are attached for prolonged periods. To some extent the newborn marsupial resembles a eutherian embryo, and the main post partum growth occurs during a long period of lactation. Eutherian mothers, in contrast, have a placenta and the fetus grows in a uterus, with the newborns exhibiting greater development and size. In eutherians the period of lactation compared with that of gestation is considerably shorter than in marsupials.

Suckling methods also differ among these mammalian subclasses. Lactating monotremes, platypus and echidna, do not have nipples; the milk is secreted onto the skin from a milk patch, which is comprised of around 100 small holes and is located in two areas on the abdomen. The neonates lick to ingest the milk which accumulates on the hairs. The female red kangaroo, a marsupial, has four nipples, only one of which begins milk secretion immediately after her newborn suckles on it. The neonate continues to suck on the teat for approximately 10 weeks after birth, and then sometimes releases it. He/she sometimes shows its face from inside it's mother's pouch at 25 weeks post partum and begins to leave the pouch at 28 weeks. He/she permanently leaves the pouch at 35-45 weeks but continues to suckle from outside the pouch until 50 weeks. Some marsupials, such as kangaroos, can produce two kinds of milk differing in composition, from two different teats, at the same time; one is a small teat supplying milk to a newborn young, while the other is a large teat which supplies milk to a much older animal that is still suckling from outside the pouch [8]. Eutherian neonates suckle their mother's milk from a nipple, similar to marsupial newborns.

Mammals have evolved via synapsids, therapsids, cynodonts, and mammaliaforms after the divergence of the common ancestor of synapsids and sauropsids. The time when mammaliaforms evolved into primitive mammals was estimated to be approximately 160 MYBP, during the late Jurassic, based on the acquisition of diphyodont and epipubis characteristics in the fossil records [9-12]. During the evolution of synapsids to mammals, the following changes occurred: locomotion from lizard-like flat movement to upright movement, acquisition of diaphragmatic breathing, acquisition of a thermal exchange system in the nasal cavity for cooling and moisture retention, and strengthening of chin muscle tissue. The mammary gland, which secretes milk, has also evolved from an ancestral gland. Olav Oftedal has suggested that the mammary gland evolved from an apocrine gland, but not from other glands such as the eccrine and sebaceous glands, based on the following observations [9-12]. Milk fat triglycerides are biosynthesized near the basal membrane within mammary epithelial cells, move to the cell membrane, and then move outside by breaking through the membrane. During this process, the fat droplet is enveloped by a membrane, called the fat

particle membrane, which originates from the cell membrane. Mammary and apocrine glands both share this type of fat extracellular transport system. Monotreme mammary glands, like apocrine glands, are associated with hair follicles, whereas marsupial and eutherian mammary glands are not. In the fetus of the opossum, a marsupial, the mammary gland primordium develops below the skin surface and then inverts to be a nipple. Although it is observed that the mammary gland primordium is associated with hair, the hair peels off when inversion occurs. This suggests that in the mammalian ancestor the mammary glands had been associated with hair follicles.

The present milk components must have been acquired during the evolution of mammary glands. In Oftedal's hypothesis, lactation originally began by providing moisture to the embryo in the egg during the egg-laying process and then evolved also to provide anti-infection factors and finally to supply the nutritional constituents, along with the anti-infection components, to the suckling neonate after hatching or birth [9-12]. Some of the milk components, including casein, β-lactoglobulin, α-lactalbumin, triglycerides, lactose, and milk oligosaccharides, are biosynthesized by the epithelial cells of lactating mammary glands, while others, including serum albumin and immunoglobulin, are transported from the blood circulation. This suggests that selective transport of some components into the mammary cells would have occurred during their evolution, while the milk-specific components, especially proteins, had evolved from ancestral components.

The evolution of milk components has been elucidated for only a few. For milk fat to be secreted as a fat particle enveloped by a membrane, the association of three proteins, butyrophilin, xanthine oxidoreductase and adipophilin, is required near the cell membrane. When butyrophilin or xanthine oxidoreductase is reduced or eliminated from mouse mammary cells via knockout of their genes, mice fail to produce normal milk and triglycerides accumulate within the secretory mammary cells [12,13,14]. It can be hypothesized that these proteins would have started to associate within mammary cells during their evolution. Kawasaki et al. speculated that αs and β caseins were derived from ancestral proteins in ameloblasts, which are involved in the mineralization of tooth enamel, while κ-casein evolved from another protein in soft connective tissue, preventing the spontaneous precipitation of calcium phosphate [15]. Caseins are associated with calcium phosphate, similar to the hypothetical ancestral proteins.

3. Evolution from lysozyme to α-lactalbumin: the origin of lactose and milk oligosaccharides

It is known that α-lactalbumin (α-LA), a milk protein, resembles c-type lysozyme, which is found in chicken egg white, with respect to its primary and tertiary structures [16].

Lysozyme acts as an anti-infection protein by hydrolyzing bacterial cell wall peptidoglycans. In contrast, α-LA associates with β1-4-galactosyltransferase 1 (β4GalT 1) within mammary cells to form a subunit of lactose synthase. The activity of β4GalT 1 is found in many tissues other than mammary glands where it usually transfers Gal from UDP-Gal to the non-reducing GlcNAc residue of glycolipids or glycoproteins [17]. α-LA, which is found only in milk or mammary glands, changes the substrate specificity of β4GalT 1 from GlcNAc to Glc, indicating that it is a modifier. As α-LA is a “new” protein, found only in mammalian milk, it must have evolved from an “old” protein, lysozyme, which is found in other vertebrates and in insects. This evolution is generally considered to be a result of duplication of the lysozyme gene.

It can be hypothesized that the evolution of α-LA from lysozyme had proceeded via an intermediate bifunctional protein which had both α-LA and lysozyme functions [18]. In 1974, this protein had been reported to be found in milk of the echidna of Kangaroo Island [19], but this could not be confirmed [20], and the existence of this protein is negated at present. Because of the low concentration of α-LA in the milks of platypus and echidna, separation and purification were difficult, but Messer et al [21, 22] finally succeeded in isolating this protein from both milks. They determined the primary structures which they then compared with each other and with those of the α-LA’s of several species of marsupials and eutherians and of lysozymes. These comparisons led to the conclusion that the echidna and platypus lineages diverged from their last common ancestor at least 50 to 57 MYBP [23], dates which were confirmed by recent analyses based on comparisons of the whole genomes of both monotremes, indicating that their divergence time was approximately 55 MYBP [6].

The amino acid sequences of the platypus and echidna α-LAs and bovine lysozyme are compared in Fig. 1. The numbers of residues are derived from the sequences of the monotreme proteins. The following is noteworthy:

□) Platypus and echidna α-LAs both retained Glu-35, which corresponds to one of the two catalytic sites of lysozyme, namely Glu-35 and Asp-53.

□) Platypus and echidna α-LAs both have Asn-44, Ala-108, and Trp-112, which correspond to the permanent residues of lysozyme. These residues, which are related to the ability of lysozyme to the lysozyme substrate, have been lost in the α-LAs of marsupials and eutherians [21,22].

These observations support the notion that monotreme α-LAs are more similar to the ancestral protein, lysozyme, than are the α-LAs from other species [21, 22, 23]. Although platypus and echidna α-LAs retained Glu-35, which is one of the catalytic sites of lysozyme, both of these proteins have lost Asp-53. If this residue had been retained in both, they might have functioned as lysozyme as well as α-LA. As aforementioned, one could speculate that

there had been a potential bifunctional protein at one time. Fig. 2 shows a virtual scheme of the evolution of lysozyme to α-LA [3], suggesting that mutations occurred in lysozyme at positions that were not catalytic sites, to produce the hypothetical bifunctional protein, after which successive mutations occurred at the positions of the catalytic sites of lysozyme to produce the current α-LA. One can imagine that the monotreme α-LAs correspond to the intermediate stage of evolution from the bifunctional protein to the α-LAs found in other later species [3].

After α-LA had been acquired in the primitive mammary glands of the ancestor, and this α-LA then associated with β4GalT 1, biosynthesis of the lactose unit could begin. Notably, mammary cells exhibit the activities of several glycosyltransferases, for which lactose is a substrate [24]. As mentioned previously, milk oligosaccharides, which are found in the milk/colostrum of many mammals, have a lactose unit at their reducing ends and also contain *N*-acetylglucosamine (GlcNAc), galactose (Gal), fucose (Fuc), *N*-acetylgalactosamine (GalNAc), *N*-acetylneuraminic acid (Neu5Ac), and/or *N*-glycolylneuraminic acid (Neu5Gc) residues. These monosaccharide residues can enzymatically be attached to the lactose unit and to each other; consequently milk oligosaccharides are biosynthesized by the actions of several specific glycosyltransferases [24].

The acquisition of α-LA is therefore the key to the evolutionary appearance of lactose as well as of milk oligosaccharides [1,2,3]. To obtain a significant concentration of lactose and of oligosaccharides in milk, the transfer of glucose from the blood circulation to the mammary cells via the basal membrane, as well as the concentrations of the donor sugar nucleotides, must also have increased during evolution. We have proposed that the ratio of lactose to milk oligosaccharides in milk is affected by the relative expression levels of α-LA to that of glycosyltransferases in the mammary glands of lactating mammals [1,2,3]. When the expression rate of α-LA is greater than that of the glycosyltransferases, lactose would predominate over milk oligosaccharides in milk/colostrum, as in most eutherians [1,2,3]. Conversely, when the expression of glycosyltransferases is greater than that of α-LA, milk oligosaccharides would be dominant, as in the milk/colostrum of living monotremes and marsupials [1-5]. The fact that in the milk of monotremes, which have retained primitive features of the common ancestor of extant mammals, oligosaccharides predominate over lactose, suggests that milk oligosaccharides also predominated in the milk-like secretions of this ancestor, which would have been due to low expression of α-LA in the primitive mammary glands of the ancestor [1,2,3].

In this paper we review studies on milk oligosaccharides in monotremes, marsupials, and eutherians, and also discuss the selection pressures relating to the ratio of lactose to milk oligosaccharides in milk, with respect to the survival strategies of each of the mammalian

subclasses.

Modification of the acceptor of β4GalT 1 from GlcNAc to Glc by interaction with α-LA can be explained by wobbling of substrate recognition caused by loose substrate specificity of β4GalT 1 and a molecular switch triggered by α-LA [25]. The steric configuration of the binding pocket of β4GalT 1 is modified by interaction with α-LA. In *in vitro* experiments, the potential donor substrate of β4GalT 1 is modified from UDP-Gal to UDP-GalNAc or to UDP-Glc, depending on the concentration of α-LA [26,27]. However, the product, GalNAcβ1-4GlcNAc (LacdiNAc), has been found in its free form only at trace levels in bovine colostrum [28], or as the carbohydrate moiety of glycoproteins of bovine milk/colostrum [29,30,31], while Glcβ1-4GlcNAc has not been found in any natural source, including milk/colostrum. In addition, another potential acceptor, myo-inositol, can be utilized by β4GalT 1 through interaction with α-LA [32]. The product, Galβ1-6myo-inositol, has so far been found only in rat milk [33,34] and in porcine colostrum (T. Saito, unpublished result). The myo-inositol may be derived from the animals' diet. LacdiNAc and Galβ1-6myo-inositol have been found in only in a few cases, in contrast to the ubiquitous presence of lactose and oligosaccharides in milk/colostrum. We hypothesize that positive selection had occurred for lactose and milk oligosaccharides, but not for LacdiNAc and Galβ1-6myo-inositol.

The α-LA molecule has several critical regions. Saccharide binding is achieved through the cleft region of α-LA. The aromatic cluster Ⅰ (AC1) within α-LA, specifically Leu-100, facilitates its interaction with β4GalT 1 and is critical for lactose synthesis activity. The crucial nature of this AC1 site is highlighted by the fact that mutation of the AC1 flexible loop region (as occurs in otariids like the fur seal) or deletion (as occurs in the walrus) suppresses the association with β4GalT 1 and leads to a milk devoid of lactose [35]. The cell biology, biochemistry, genetics as well as the extrinsic and intrinsic factors of lactose synthesis were comparatively reviewed across some species [36].

4. Monotreme milk oligosaccharides

Studies on the milk oligosaccharides of monotremes were initiated by Knowles Kerry and Michael Messer, who had worked in the Gastroenterological Research Department of the Royal Melbourne Childrens Hospital. Based initially on the results of simple paper chromatography and later on Sephadex G-15 gel filtration analysis of the carbohydrates extracted from platypus and echidna milk, they showed that milk oligosaccharides predominate over lactose in both milks; this finding was published in "Science" Journal in 1973 (Fig. 3) [37]. The paper contradicted the longstanding view that lactose was always the dominant carbohydrate in milk, which may have come as a surprise to some of the scientific

community. The echidna milk had been obtained by Dr. Mervyn Griffiths from two wild animals caught on Kangaroo Island and New South Wales, respectively. The platypus milk was collected from 12 animals captured in the upper Shoalhaven River, New South Wales.

The gel filtration profiles of monotreme milk carbohydrates are shown in Fig. 4. The results showed sialyllactose and fucosyllactose as being the predominant saccharides in echidna milk, while difucosyllactose was dominant in platypus milk together with a small amount of fucosyllactose [37]. Both milks also contained small amounts of lactose. Fucosyllactose and difucosyllactose were later characterized as Fucα1-2Galβ1-4Glc (2'-FL) and Fucα1-2Galβ1-4(Fucα1-3)Glc (DFL), respectively, later, in a ^{13}C NMR study [38]. The sialyllactose in the platypus milk was unique because it had an *O*-acetyl group in the Neu5Ac residue, and was later characterized as 4-*O*-acetyl-Neu5Acα2-3Galβ1-4Glc (4-OAc-3'-SL) by Messer [39], which was confirmed by Hans Kamerling et al. using ^{1}H-NMR spectroscopy [40]. As shown in Fig. 4 platypus milk contained higher neutral oligosaccharides which eluted prior to difucosyllactose in gel filtration. These were characterized as shown in Fig. 5 by collaboration between Akira Kobata, Junko Amano of Tokyo University, and Messer [41], and shown to contain the core units of lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc, LN*n*T), lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc, LNT), and lacto-*N*-neohexaose [Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc, LN*n*H], which have been found in the milk of some eutherians including humans [1-5]. It is also characteristic that many of them contained Lewis x [Galβ1-4(Fucα1-3)GlcNAc] or Lewis y [Fucα1-2Galβ1-4(Fucα1-3)GlcNAc] units. The milk oligosaccharides of monotremes were found to be more similar to those of eutherians than of marsupials [1-5], as mentioned below.

Further, it was shown that platypus milk contains sialyl oligosaccharides which eluted earlier during the gel filtration (see Fig. 4). These oligosaccharides remained uncharacterized until 2015 when their structures were published in by Urashima et al [42], as shown in Fig. 5. Each oligosaccharide was purified using normal-phase high-performance liquid chromatography (HPLC) using an Amide-80 column, and characterized using ^{1}H-NMR and MALDI-TOF mass spectrometry. The oligosaccharides characteristically included the core units of lactose, LN*n*T, or LN*n*H, and also contained Lewis x or sialyl Lewis x [Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc]. Neu5Ac had a 4-*O*-acetyl group, similar to echidna 4-OAc-3'-SL, indicating that the presence of 4-*O*-acetyl Neu5Ac is a specific feature of monotreme sialyl oligosaccharides; it has been reported, however, that bovine colostrum contains a trace level of *O*-acetylated 3'-SL [28].

The oligosaccharides separated from milk of the Tasmanian echidna, a subspecies, were characterized in 2014 by Olav Oftedal of the Smithsonian Environmental Institution and his Tasmanian coworkers [43]. The dominant saccharides were Galα1-3(Fucα1-2)Galβ1-

4(Fucα1-3)Glc (B pentasaccharide) and Galα1-3(Fucα1-2)Galβ1-4Glc (B tetrasaccharide), whereas lactose was a minor saccharide similar to the observation in other echidnas and the platypus. This milk also contained 2'-FL, DFL, 4-OAc-3'-SL, 4-*O*- acetyl Neu5Acα2-3Galβ1-4(Fucα1-3)Glc, and Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc（lacto-*N*-fucopentaose III, LNFP III）with trace amounts of Neu5Acα2-3Galβ1-4Glc (3'-SL), di-*O*-acetyl 3'-SL, and 4-OAc-3'-SL sulfate. In LNFP III, another core unit of LN*n*T other than the lactose core was detected, though at a low concentration. Although Tasmanian echidna milk contains oligosaccharides that contain B antigen [Galα1-3(Fucα1-2)Gal], this type had not been found in the milk samples from Kangaroo Island or New South Wales echidnas, illustrating the heterogeneity of milk oligosaccharides among these subspecies.

What is the specific physiological significance of the 4-*O*-acetylation of Neu5Ac in monotreme milk oligosaccharides? Are milk oligosaccharides and lactose absorbed and digested in the small intestine of the young monotremes? Biochemical experiments were performed relating to the digestion of saccharides by several glycosidases in homogenates of small intestinal tissues of two echidna neonates [44]. Neutral β-galactosidase activity was absent but the activities of several acidic glycosidases, including β-galactosidase, α-L-fucosidase and neuraminidase were observed. Thus monotreme neonates do not have the small intestinal brush border neutral β-galactosidase (lactase) activity which normally functions in the digestion of milk lactose in eutherian neonates. It has been suggested, therefore, that the monotreme milk oligosaccharides, like marsupial milk oligosaccharides (see below) enter the small intestinal cells via pinocytosis or endocytosis and are then transferred to lysosomes by mechanisms, and hydrolyzed by the actions of the above-mentioned acid glycosidases (see Fig. 6A). It can be assumed that the resulting monosaccharides would mainly be utilized as an energy source by monotreme neonates, although sialic acid could be important for brain growth [1,5,44].

We have hypothesized that the presence of 4-*O*-acetylated Neu5Ac in monotreme milk may be related to its anti-infection function, based on the secretion of the milk onto the unsterile skin by nipple-less mammary glands [5, 43]. If the milk components could be utilized as a nutritional source for the growth of bacteria on the skin, this would be deleterious to the young and the mother. As bacterial sialidases do not have substrate specificity for sialyl oligosaccharides containing 4-*O*-acetyl Neu5Ac [45], most of the sialyl oligosaccharides in monotreme milk could not be an energy source for the growth of bacteria. It is noteworthy that in a digestion test using a homogenate of the small intestine of an echidna neonate, 4-OAc-3'-SL was converted to Neu5Ac, Gal and Glc, suggesting the presence of esterase activity in this tissue [44] which would remove the acetyl group from Neu5Ac. Therefore the sialyl oligosaccharides containing 4-*O*-acetyl Neu5Ac in the milk of the mother could be

utilized by her neonates, but could not be utilized by bacteria growing on the skin of the mother. In addition, *in vitro* experiments have shown that sialyllactose or fucosyllactose can inhibit the attachment of *Campylobacter jejuni* to epithelial cells [46,47] thus exhibiting a likely anti-infection effect against any pathogenic microorganisms present on the skin above the mammary glands of the mother and in the colon of the neonate after the consumption of milk.

5. Marsupial milk oligosaccharides

Detailed studies on the milk oligosaccharides of this group of mammals were begun in 1977 by Messer et al on the Eastern grey kangaroo [48] and in 1979 on the tammar wallaby, another macropod marsupial [49]. It was shown that the milk of these marsupials can contain very high concentrations, up to 14% of unknown oligosaccharides rich in galactose, along with small amounts of lactose, depending on the time of lactation. The gel filtration profiles of the carbohydrates separated from the milk of a tammar wallaby, collected at 3, 26 and 32 weeks after parturition, are shown in Fig. 7. The numbers on each peak indicate the number of monosaccharide residues contained within the saccharide, in which No. 2 therefore corresponds to lactose. It is apparent that lactose is only a minor component of the milk, with higher oligosaccharides, ranging up to at least octasaccharides being dominant during mid lactation.

A structure study was performed by Howard Bradbury (ANU, Canberra) and Messer et al on the neutral milk oligosaccharides of a tammar wallaby using gel filtration for purification and mainly ^{13}C-NMR techniques for characterization [50-53]. Tammar wallaby milk oligosaccharides could be grouped into two series. The major series comprised linear oligosaccharides, expressed as (Galβ1-3)$_{n=1\sim6}$Galβ1-4Glc, while the minor series comprised branched oligosaccharides such as Galβ1-3(GlcNAcβ1-6)Galβ1-4Glc (lacto-*N*-novotetraose), Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc (lacto-*N*-novopentaose I) and Galβ1-3 Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc.

Tadasu Urashima, who received samples of freeze-dried marsupial milk carbohydrates from Messer, studied neutral and acidic oligosaccharides of the red kangaroo [54], brushtail possum [55], koala [56], wombat [57], eastern quoll [58] and tiger quoll [59]. The separations were finally performed for neutral oligosaccharides using HPLC on a graphite carbon column, and for acidic saccharides using normal phase HPLC on an Amide-80 column after anion exchange chromatography. Each oligosaccharide was characterized using ^{1}H-NMR and MALDI TOFMS. Fig. 8 show the structures of the major linear series of the neutral and of the acidic milk oligosaccharides of the tammar wallaby and red kangaroo, respectively. The acidic oligosaccharides have non-reducing α2-3 linked Neu5Ac or sulfated Gal at OH-3,

which is linked to linear β1-3 galactosyl oligosaccharides. Although it seems that linear β1-3 galactosyl oligosaccharides, other than the trisaccharide, occur only in marsupials, trace levels of tetra or pentasaccharides have recently been detected in the colostrum of some eutherians including cows [60], water buffalos [60,61], and or goats [60], However, the concentrations of these linear oligosaccharides are high in the milk of marsupials, but very low in the colostrum of these few species of eutherians.

The branched oligosaccharides (see Fig. 9) are minor constituents in the milks of tammar wallaby, red kangaroo and brushtail possum, but major in the milks of the carnivorous eastern quoll and tiger quoll, indicating that there is heterogeneity in the predominance of linear or branched oligosaccharides among marsupial species. The most dominant branch-type oligosaccharides, lacto-*N*-novopentaose I and its sialyl derivatives, have been found in the milks/colostra of cows [28,62,63], horses [63,64], goats [63], sheep [63], camels [63,65,66], and pigs [63], while trace amounts of its sialyl and fucosyl derivatives have been found in human milk [67]. Thus these saccharides are common to the milks of both marsupials and eutherians. The neutral branched oligosaccharides are Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc, Galβ1-3Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc, Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-3Galβ1-4Glc and Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc, while the acidic oligosaccharides have non-reducing α2-3 or 2-6 linked Neu5Ac or sulfated Gal at OH-3, linked to the neutral core units. It is noteworthy that Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc and its sialyl derivatives are constructed from Galβ1-3Galβ1-3Galβ1-4Glc as a scaffold and two units of *N*-acetyllactosamine (Galβ1-4GlcNAc), structures which are unique to marsupial milk oligosaccharides. High molecular weight structures, which further extend longitudinally, have been found in tiger quoll milk [59]. In contrast, LN*n*T, LN*n*H, *para* lacto-*N*-neohexaose Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc, *para* LN*n*H) and their sialyl derivatives, which have been identified in the milks of many eutherian species, have not been found in any marsupial milk [1-4]. This may be due to the absence of β1-3*N*-acetylglucosaminyltransferase (iGnT) which transfers GlcNAc from UDP-GlcNAc to the OH-3 position of the Gal residue of lactose in the mammary glands of lactating marsupials, resulting in a characteristic difference in the structures of the milk oligosaccharides of marsupials and eutherians.

Among the marsupial milks studied, only koala milk was found to contain fucosyl oligosaccharides such as {Galβ1-3[Galβ1-4(Fucα1-3)GlcNAcβ1-6]Galβ1-4Glc and Neu5Acα2-3 Galβ1-3[Galβ1-4(Fucα1-3)GlcNAcβ1-6]Galβ1-4Glc} [56]. As the activity of acidic α-L-fucosidase as well as acid β-galactosidase, β*N*-acetylglucosaminidase and neuraminidase, has been found in the small intestine of tammar wallaby neonates [68,69],

one can speculate that fucosyl oligosaccharides were present in the milks of the ancestral marsupials, but have since been lost in the milks of most of the extant marsupial species.

The hypothetical biosynthetic pathways for the neutral milk oligosaccharides of eastern quoll milk are shown in Fig. 10. The activities of three glycosyltransferases, β1-4-galactosyltransferase (β4GalT), β1-3-galactosyltransferase (β3GalT) and β1-6-*N*-acetylglucosaminyltransferase (IGnT), were detected in the mammary glands of lactating tammar wallabies [70,71]. The biosynthesis for all structures in Fig. 10 can be deduced from the substrate specificities of these three transferases. The β3GalT can transfer Gal to the non-reducing end of the linear unit, while IGnT can transfer GlcNAc to OH-6 of the penultimate Gal of Galβ1-3Galβ1-4Glc and Galβ1-3 Galβ1-3Galβ1-4Glc.

Can large amounts of milk oligosaccharides be utilized as nutrients by marsupial neonates? Histochemical studies with specific stains for neutral and acid β-galactosidases have shown that a neutral intestinal lactase is completely absent from the brush borders of the villi of the small intestine of the suckling tammar wallaby [68,69,72]. Instead, a very active acid β-galactosidase is present intracellularly, probably located within the lysosomes and supranuclear vacuoles [68,69,72]. This indicates a very different mechanism for the utilization of milk saccharides by suckling macropod neonates compared with newborn eutherians. When the eutherian neonate consumes its mother's milk, the predominant saccharide, lactose, is split into glucose and galactose by an intestinal lactase (neutral β-galactosidase), which is located in the membrane of the microvilli of the small intestinal brush border, and these monosaccharides are transported into the enterocytes via specific mechanisms (see Fig. 6B). Glucose enters the circulation for utilization as an energy source, while galactose is converted to glucose in the liver to be utilized as an energy source as well. This mechanism, however, is evidently absent in the suckling tammar wallaby young because of absence of the neutral lactase, and of other glycosidases such as neuraminidase and fucosidase, from the small Intestinal brush border.

The acid β-galactosidase can hydrolyze lactose as well as the β1-3-galactosides in tammar milk, thus differing from the eutherian neutral lactase, which hydrolyzes only lactose. Additional biochemical experiments have shown that the neutral lactase is also absent in two other macropod species, the red kangaroo and the grey kangaroo [68,69]. In contrast, several acid glycosidases including β-galactosidase, *N*-acetylglucosaminidase, α-L-fucosidase and neuraminidase, were readily detected in the tammar wallaby small intestine [68,69]. These results show, therefore, that the oligosaccharides of macropod milk cannot be hydrolyzed at the brush border of the macropod small intestine and suggest that, for digestion, they must first be transported into the enterocytes where they can then be hydrolyzed to their constituent monosaccharides by the lysosomal acid glycosidases. The mechanism by which

this transport takes place is still unknown, but it has been suggested that pinocytosis or endocytosis is the most likely [68,69,72] (see Fig. 6A). Notably, this mode of absorption and lysosomal digestion would be relatively slow, which would explain lactose intolerance in infant kangaroos and other macropods. It is well-known that pouch-young macropods ("joeys") cannot be bottle-fed with cow's milk but need to be fed special low-lactose milk formulas such as Biolac and Wombaroo, to survive.

Studies conducted to date suggest that among marsupials the brush border intestinal lactase may be completely absent only in kangaroos, wallabies and other macropods. Very young brushtail possums aged under 125 days resemble macropods in that the activity of this lactase is low [73], whereas acid β-galactosidase activity is high [73], but as the animals age, the situation reverses, with the result that older suckling possums have intestinal lactase activity similar to that found in eutherians. The reversal of the activities of the galactosidases is accompanied by changes in the milk carbohydrates, mainly from oligosaccharides to free lactose, whose concentration reaches approximately 5% prior to weaning [73]. The changes in milk carbohydrates during lactation observed in brushtail possums were also seen in ringtail possums [74]. Furthermore, preliminary experiments have suggested that intestinal neutral lactase activity is present in two other marsupial species, the Southern brown bandicoot and the eastern quoll [69].

The presence of intestinal lactase activity in older suckling neonates of brushtail possums suggests that the neutral lactase gene was acquired in therians, the common ancestors of marsupials and eutherians, only after their divergence from the monotremes, which do not have the neutral intestinal lactase. From the above observations it would seem that in marsupial neonates the choice between utilizing milk oligosaccharides by lysosomal digestion or utilizing milk lactose by the action of a neutral intestinal lactase may be governed by some selective advantages. The younger suckling brushtail possum utilizes the oligosaccharides, while the older suckling brushtail possum depends on lactose. As the molecular weights of these oligosaccharides are greater than that of lactose, the osmotic pressure exerted by them per gram weight in an aqueous solution would be lower than that of lactose. Even though at one time point during lactation tammar wallaby milk contains approximately 14% of carbohydrate [49], its osmotic pressure would not be too high, as this carbohydrate consists of a mixture of higher molecular weight oligosaccharides ranging from a trisaccharide to at least octasaccharides. This ensures that the high carbohydrate concentration in the milk is not too stressful, in terms of osmotic pressure, for the small intestine of the suckling marsupial neonate. This would constitute a selective advantage of the macropod lysosomal digestion mechanism. Another advantage may be the supply of Neu5Ac, GlcNAc and sulfate to the suckling neonates. During evolution the macropod

marsupials may have lost the neutral intestinal lactase as a result of selection pressure.

The ratio of oligosaccharides to lactose in milk would be determined by the ratio of the expression rates of glycosyltransferases genes to that of α-lactalbumin in lactating mammary cells [1,2,3]. The above phenomenon observed in the brushtail possum suggests that an increase in the expression of α-lactalbumin in the mammary glands of the mother can be linked to the appearance of neutral lactase activity in the small intestine of the suckling neonates [73]. As already noted, in macropod marsupials the lysosomal digestion system for oligosaccharides was selected instead of the eutherian mechanism for lactose utilization. The exceptions among other marsupials were the brushtail possum and probably some other species, in the late suckling stage.

6. Human milk oligosaccharides (HMOs)

Eutherian milk or colostrum, unlike that of monotremes and marsupial milk, usually contains only small amounts of oligosaccharides, lactose being the dominant saccharide [75]. The predominance of lactose in milk is likely to have been acquired by the enhancement of the expression level of the α-LA gene and also by an increase in the transport of glucose into the mammary cells [1-3]. It can also be considered that an increase in lactose concentration within those cells may have stimulated the secretion of the biosynthesized milk components as a result of an increase in the intracellular osmotic pressure.

The ratio of milk oligosaccharides to lactose in eutherian milk varies depending on the species [1-5]. As described above, suckling eutherian neonates have small intestinal lactase activity, and thus lactose in milk is split into glucose and galactose, which are absorbed and enter the circulation (see Fig. 6B). Whether milk oligosaccharides are similarly digested and absorbed in the small intestine of non-human eutherian neonates is still unclear. The milk of rats contains a significant amount of sialyllactose along with the dominant lactose [76]; strong neuraminidase activity, probably originating from lysosomes, has been detected in the rat small intestine [77], suggesting that in the neonatal rat milk sialyllactose enters the small intestinal enterocytes by pinocytosis or endocytosis as in monotremes and marsupials (see above) and is then digested to glucose, galactose and sialic acid by lysosomal enzymes. Although the neonates of eutherians, including humans, usually obtain significant energy from lactose through digestion by intestinal lactase, suckling rats would also obtain some energy from the sialyllactose by lysosomal digestion. In addition they would obtain sialic acid, which could be important for brain development and other functions [78].

In human milk the ratio of milk oligosaccharides to lactose averages 1:4 [79, 80]. There is a consensus that in human infants the major part of the milk oligosaccharides cannot be

digested and absorbed in the small intestine and thus reach the colon [81,82,83], presumably because of the absence of the requisite brush border glycosidases such as fucosidases and neuraminidase.

Human milk oligosaccharides (HMOs) have been studied more intensely than the milk oligosaccharides of other mammals. These studies have provided detailed characterizations of HMOs with respect to their chemical structures [79, 80, 84, 85], concentrations [86, 87], and biological functions [88], which have been subjected to numerous reviews [79, 80, 84, 85, 86, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97]. The concentration of HMOs is estimated to be 12-13 g/L in mature milk and 22-24 g/L in colostrum, representing the third largest solid component in the human milk after lactose and lipids [97]. Structural studies of HMOs were first conducted by R. Kuhn in the 1950s and then by J. Montreuil, A. Kobata, H. Egge, G. Strecker, I. Yamashina and R. Cummings, each of whom conducted pioneering research in glyco-chemistry [79,80,84,85,93]. To date, more than 250 oligosaccharides have been purified, of which 169 have been characterized [80] and are classified into 20 series based on their core structures; these do not contain fucose and Neu5Ac residues (Table 1) [80]. The potential biosynthetic pathways for the core structures are shown in Fig. 11 [80], in which it is hypothesized that they involve combinations of the actions of the following four glycosyltransferases: β1-3*N*-acetylglucosaminyltransferase (iGnT), which transfers GlcNAc from UDP-GlcNAc to OH-3 of the Gal residue of lactose or LacNAc unit; β1-6*N*-acetylglucosaminyltransferase (IGnT), which transfers GlcNAc to OH-6 of the Gal residue of GlcNAcβ1-3Galβ1-4Glc; β1-3galactosyltransferase (β3GalT), which transfers Gal from UDP-Gal to OH-3 of non-reducing GlcNAc; and β1-4galactosyltransferase (β4GalT), which transfers Gal to OH-4 of non-reducing GlcNAc. When an oligosaccharide has Galβ1-3GlcNAc (lacto-N-biose I, LNB) at its non-reducing end, it cannot be further extended. Although both β3GalT and β4GalT have substrate specificity for β1-3-linked GlcNAc, only β4GalT has specificity for β1-6-linked GlcNAc. However, one cannot exclude the possibility that another IGnT, which transfers GlcNAc to the OH-6 position of Gal, linked to Glc(NAc) of Galβ1-4GlcNAcβ1-3Galβ1-4Glc(NAc), can function in the biosynthesis of some core units, as shown by the dotted line in Fig. 11.

Fucosyl HMOs are synthesized by α1-2fucosyltransferase (FUT2) or α1-3/4fucosyltransferase (FUT3) to form H type 1 (Fucα1-2Galβ1-3Glc), Lewis a [Galβ1-3(Fucα1-4)GlcNAc], Lewis b [Fucα1-2Galβ1-3(Fucα1-4)GlcNAc], Lewis x [Galβ1-4(Fucα1-3)GlcNAc], and Lewis y [Fucα1-2Galβ1-4(Fucα1-3)GlcNAc], while sialyl HMOs are synthesized by α2-3 or α2-6-sialyltransferases to form Neu5Acα2-3Galβ1-3GlcNAc, Neu5Acα2-6Galβ1-4GlcNAc, or Galβ1-3(Neu5Acα2-6)GlcNAc. Although it has been reported that only Neu5Ac but not Neu5Gc, is present in sialyl HMOs, a recent study has

suggested that Neu5Gc, originating from the diet of the mother, can be found in HMOs at very low concentrations [98].

Kellman et al., recently, used a systems biology framework that integrated glycan and RNA expression data to construct an HMO biosynthetic network and predict glycosyltransferases involved [99]. They further explored selected enzyme activities through kinetic assay and their co-regulation through transcription factor analysis.

HMOs are more varied than the oligosaccharides of other eutherian milks. The main reason is that HMOs contain both type 1 and type 2 oligosaccharides, which have LNB or LacNAc, respectively, unlike the milk oligosaccharides of most other eutherian species [79,80,84]. In human milk the type 1 oligosaccharides predominate over type 2, whereas the milks of other eutherian species mostly contain only type 2 oligosaccharides, or else in a few species the type 2 predominates over type 1 [79,80,96]. The predominance of type 1 in milk appears to be a human-specific feature. As described below, we speculate that the acquisition of the type 1 predominance in human milk may have been a selective advantage with respect to the establishment of the bifidus flora in the human colon [80, 96].

HMOs profiles vary between donors, and there are significant differences depending on the donors' Secretors/non-Secretors status or Lewis blood groups [79,80,84]. Secretor donors have ABO human blood group-type secretion in body fluids, which non-Secretor donors do not have. The milks of Lewis-positive Secretor donors, which comprise 80% of donors in many ethnic groups, contains all oligosaccharides, while the milks of Lewis-positive non-Secretor donors, which comprise approximately 15%, mostly contain little or no 2'-FL (Fucα1-2Galβ1-4Glc), LNFP-I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc), and LNDFH-□ [Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc], etc, which have a non-reducing H antigen (Fucα1-2Gal). These types of HMOs, however, are the most dominant in the milks of Secretor donors. The milks of Lewis-negative donors, which comprise approximately 5% of donors in the world, usually contains no LNFP-□ [Galβ1-3(Fucα1-4)GlaNAcβ1-3Galβ1-4Glc] and LNDFH-□, etc, which have a Fucα1-4 residue in their structures. The concentrations of representative HMOs were determined for each donor group [86,87]. Some variations were observed depending on the ethnic group [79,80,85,100]. It is also characteristic of human milk that the concentration of neutral oligosaccharides, especially fucosyl HMOs, is higher than that of sialyl oligosaccharides [79,80,85].

How have HMOs provided evolutionary advantages for humans, even though they do not serve as a significant energy source for nursing infants? We hypothesize that humans have taken advantage of HMOs to establish symbiosis with colonic bacteria. As humans have relatively immature newborns, the mothers need to fortify their milk with prebiotic agents to stimulate the growth of beneficial (probiotic) microbes in infant colon.

The genus *Bifidobacterium* is one such beneficial taxon, as it produces physiologically active compounds (e.g., acetic acid, lactic acid, and aromatic lactic acids) that are important in the maintenance of gut homeostasis and the development of the host immune system [101,102,103,104]. *Bifidobacterium* sp. was first isolated by Henri Tissier at the Pasteur Institute in 1899 from breastfed infant feces. It has been historically accepted that HMOs stimulate the growth of bifidobacteria; however, the metabolic pathways of HMO degradation by bifidobacteria had not been elucidated until recently [91,92,105]. During the last 17 years, several groups have successfully identified and characterized the genes responsible for HMO-utilization in bifidobacteria. These results, together with homology-based database searches, revealed that bifidobacteria have evolved two different strategies to utilize HMOs: extracellular glycosidase-dependent and intracellular glycosidase-dependent. Interestingly, the distribution of the gene repertoire within the *Bifidobacterium* genus markedly varies not only at the species level but also at the strain level. In the following section, we summarize HMO utilization pathways in bifidobacteria with an emphasis on two representative species that avidly consume HMOs using different strategies: i.e. *Bifidobacterium. bifidum* and *Bifidobacterium. longum* subsp. *infantis*. Thereafter, we describe how HMO-related genes are differentially conserved among *Bifidobacterium* genus, species, and strains.

B. bifidum is equipped with the gene set for extracellular HMO digestion (Fig. 12). During HMO metabolism by *B. bifidum*, monosaccharides (Fuc, Neu5Ac, Gal, GlcNAc, and Glc) and disaccharides (LNB and lactose) are extracellularly liberated by a series of glycosidases. LNB is imported by the galacto-*N*-biose (GNB)/LNB-specific ABC uptake system (GltABC [GNB/LNB-BP]) and then phosphorolyzed by GNB/LNB phosphorylase (LnpA) to produce Gal-1-P and GlcNAc intracellularly [106,107]. While GlcNAc, Glc, LNB, and lactose are consumed during the growth, Fuc, Neu5Ac, and Gal remain unconsumed [108,109]. The transient and/or continuous release of the sugars into the medium during cultivation is a common feature of *B. bifidum* and constitutes the basis for its cross-feeding property. The addition of *B. bifidum* in fecal cultures significantly increased the population and relative abundance of other *Bifidobacterium* species in the fecal bacterial community [108]. The stimulatory effect was observed only in the medium supplemented with HMOs and not in the Glc-supplemented medium. Sialyllactose (SL) is another HMO that supports cross-feeding within *Bifidobacterium* [110,111]. *B. bifidum* possesses extracellular sialidase SiaBb2, by which Neu5Ac is released. While *B. bifidum* does not assimilate sialic acid and *B. breve* does not assimilate SL, *B. breve* can assimilate the released Neu5Ac. The altruistic behavior of *B. bifidum* was also seen in the culture medium containing mucin glycoproteins, as it also has extracellular

glycosidases acting on *O*-glycans (note that *O*-glycans and HMOs share structural similarity) [112,113]. The extracellular digestion strategy seems to be exclusive to *B. bifidum*, with one exception. Some strains of *B. longum* subsp. *longum* harbor lacto-*N*-biosidase (LNBase) LnbX as the sole extracellular enzyme and thereby releases LNB and lactose from LNT outside of the cell [114]. In this case also, the liberated products are shared among bifidobacterial [106].

B. longum subsp. *infantis* is equipped with the gene set for intracellular HMO digestion (Fig. 12). This subspecies imports almost all types of HMOs using ABC transporters and then sequentially degrades the imported HMOs from the non-reducing end inside the cell [115]. This phenotype is largely attributable to the presence of a 43-kbp gene cluster (Blon_2331–Blon_2361), designated as the HMO-1, that is comprised of many genes encoding for transporters and intracellular *exo*-glycosidases [91,115]. However, a β-galactosidase gene required for hydrolysis of LNT is located outside the cluster [116]. Likewise, the gene clusters required for FL and LN*n*T uptake are separately present in the genome [117,118,119]. While the HMO-1 cluster is specific to *B. longum* subsp. *infantis*, other bifidobacterial species also possess similar intracellular machinery to utilize HMOs, although their gene repertoire is limited compared to *B. longum* subsp. *infantis* [105,118]. The specificity of speculated multiple HMO transporters of *B. longum* subsp. *infantis* has not been fully elucidated, however, HMO-mediated symbiosis between bifidobacteria and infants was recently highlighted by the study of two FL transporters, 1 and 2, of *B. longum* subsp. *infantis*. The fecal abundance of FL transporter gene homologs was negatively associated with the concentration of substrate HMOs in breastfed infant feces and at the same time positively associated with bifidobacterial richness in the fecal community [117].

Fig. 13 shows the prevalence of HMO-related genes in *Bifidobacterium* at the species and strain levels, which was examined by tblastn analysis [105]. The occurrence of the genes responsible for HMO assimilation is essentially limited to infant- and infant/adult-type species, such as *B. bifidum*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. breve*, *B. catenulatum* subsp. *kashiwanohense*, and *B. pseudocatenulatum*. As mentioned above, the occurrence of extracellular glycosidase genes is limited to *B. bifidum*, except for some *B. longum* subsp. *longum* strains that have lacto-*N*-biosidase (LnbXY). HMO transporter genes, though not fully identified, occur sporadically between species and/or strains. Collectively, although there are some exceptions, the conservation and distribution of the HMO assimilation genes suggest an evolutionary adaptation of infant-type bifidobacteria to HMOs. In particular, conservation of type-1 chain (Galβ1-3GlcNAc) specific enzymes such as LnpA, GltABC, and Bga42A among

Bifidobacterium residing in the human infant colon may represent a co-evolutionary trajectory between *Bifidobacterium* and humans, mediated through type-1 chain rich HMOs.

Recent studies show that, in addition to bifidobacteria, other colonic bacteria such as *Bacteroides* species*, Lactobacillus casei*, *Akkermansia muciniphila,* and Clostridiales are also equipped with the genes responsible for degrading several HMO molecules or HMO degradants [120,121,122,123,124]. *Roseburia* and *Eubacterium* members in Clostridiales utilize a few type-I HMOs by intra- and/or extracellular digestion and produce butyrate as an end product. *A. muciniphila*, which is considered to be beneficial to human health, extracellularly degrades multiple HMO species including type-I HMOs. *L. casei*, some strains of which are commercialized, utilizes lacto-*N*-triose II and LNB. In general, the HMO utilization ability of these bacteria is not as high as bifidobacteria; nonetheless, the predominance of type-I oligosaccharides in human milk could have been a selective pressure for the formation of healthy microbiome in the infant colon. It is also noteworthy that some species of *Roseburia* can assimilate both HMOs and plant-derived glycans, suggesting an adaptive response to the transition in host diet during weaning [120].

Is co-evolution of milk oligosaccharides with colonic bacteria specific to humans or is it also found in other eutherian species? To explore this, the microflora in the feces of non-human neonates have begun be investigated. It has been found that *Enterococcus gallinarum* isolated from the feces of suckling neonatal rats could hydrolyze 3'-SL to Neu5Ac and lactose [125]. Since almost the only oligosaccharides in rat milk are 3'- and 6'-SL [125, 126], it can be concluded that the neonatal rat has a specific microflora in which *Enterococcus gallinarum* uses 3'- and 6'-SL for its growth [125]. As bifidobacteria have not been found in the feces of suckling rats, bifidus flora formation related to the metabolism of HMOs may not be applicable to rats but may nevertheless be relevant to other species. Exploration of the formation of colonic microflora in non-human species, including those of closely related species such as great apes, could well be a subject for further evolutionary study in relation to the utilization of milk oligosaccharides [127],

As mentioned above, it has been hypothesized that HMOs have biological significance other than stimulation of the growth of beneficial colonic microflora. One of these is anti-infection activity against pathogenic bacteria and viruses. It has been observed in *in vitro* experiments that the growth of group *B Streptococcu*s, which causes meningitis, was inhibited dose-dependently by the addition of HMOs at concentrations below 1.0 mg/mL [128]. When three strains of *Streptococcu*s *agalactiae* were grown in the presence of 1% HMOs and some antibiotics, the concentration for minimum growth inhibition decreased from 1/16

to 1/32 for erythromycin, gentamycin, and minocycline [129]. Several previous *in vitro* studies have demonstrated a decrease in the counts of bacteria and viruses attached to the epithelial cells cultured in a medium containing the pathogenic microorganisms and HMOs. For example in one study, when rotavirus isolated from pigs or great apes was exposed to MA-104 cells originating from tubular epithelium, the resulting infection was inhibited dose-dependently by the addition of ≧ 10 mg of HMOs [130]. However, in another study, when a different rotavirus isolated from humans was used, the infection was enhanced by the addition of HMOs, indicating that attachment of the virus to the cells is not always inhibited by HMOs [131]. On the other hand HMOs may help to enhance vaccine-induced immunity. When the ear of a mouse was vaccinated with inactivated influenza virus, the other ear being exposed to saline, the resulting inflammation was enhanced with increasing concentrations of influenza-specific serum IgG in by the feeding of 2'-FL [132].

It has been shown that following the ingestion of breast milk by human infants, most of the HMOs reach the colon, but a small portion of HMOs is absorbed by the small intestine and enters the circulation, to be excreted in the urine [133]. It has been suggested that HMOs interact with selectins or galectins which are expressed on endothelial cells or hemocytes, producing immunomodulation or reduction of inflammation. When platelets were cultured in a medium containing HMOs, and the level of the chemokine RANTES was measured after activation with thrombin and tumor necrosis factor-like soluble CD40 ligand, it was found that the level of RANTES decreased when compared with the controls without HMOs [134]. Another study showed that after ovalbumin allergic mice were sensitized with an allergen, the inflammatory cytokine level and the counts of mast cells decreased after oral feeding of 2'-FL [135].

Despite the possible immunomodulatory and anti-inflammatory effects of HMOs, the question whether they may provide a selective advantage for survival during human evolution cannot at present be answered. It is known that breastfeeding decreases the risk of development of necrotizing enterocolitis (NEC), which can causes serious damage to the colons of immature infants; an *in vivo* study suggested that HMOs can prevent NEC development. In this study NEC was induced in newborn rats by injecting an inflammatory agent into the pregnant rats; the newborns were then grouped into a DAM group, fed with their mothers' milk; an HMO group, fed with a milk replacer containing HMOs; and a control group, fed with the replacer without HMOs. After feeding for 96 h, the rats were anesthetized and their colons collected. When the degree of inflammation of the colons was scored for each group, it was found that the score of the HMO group was better than that of the control group and close to that of the DAM group. Among the HMOs, disialyl lacto-N-tetraose (DSLNT) had a specific effect of decreasing colonic damage by NEC [136]. *In vivo*

experiments suggest that the prevention of NEC by HMOs is produced by inhibition of the inflammatory TLR4 signaling pathway or by strengthening of the barrier function of colonic mucin [137,138]. Such an effect of HMOs on the prevention of NEC may have increased the survival of babies during human evolution.

The results of an *in vivo* experiment using piglets suggested that the sialic acid from sialyl HMOs orally fed to piglets could be utilized for the biosynthesis of brain sialyl glycoconjugates. After the piglets were fed for 21 days with a milk replacer containing 2-4 g/L of 3'-SL and then anesthetized, their brains were collected and the concentrations of total sialic acid and the sialic acid concentrations of gangliosides in the cerebral cortex, cerebellum, corpus callosum and hippocampus were measured, and compared with those of the controls, which had been fed with milk replacer without 3'-SL [139]. The results showed that the concentrations of total sialic acid and of sialic acid in gangliosides in the cerebellums of the piglets fed with 2 g/L 3'-SL were dose-dependently higher than those in the controls, while the total sialic acid concentration in the corpus callosum was also higher in the 2 g/L 3'-SL groups than in the controls. Thus the presence of sialyl HMOs in human milk may have been advantageous for the development of the human brain.

In summary, many biological effects of HMOs have recently been observed in *in vivo* and *in vitro* experiments; it is possible that these effects may have conferred a selective advantage for the survival of infants during human evolution.

7. Milk oligosaccharides among carnivorous species: a case in which oligosaccharides predominate over lactose in eutherian mammals

As previously mentioned, we have hypothesized that the milk oligosaccharides, as well as lactose, appeared as a result of the acquisition of α-lactalbumin in the common ancestor of extant mammals, and that milk oligosaccharides predominated over lactose in the earlier stages of mammalian evolution [1-5]. This feature was inherited by living monotremes and marsupials whereas in most eutherian species lactose became the dominant saccharide in milk and a significant energy source for suckling neonates; this was the result of a significant enhancement in the expression level of α-lactalbumin in eutherian lactating mammary cells, and was accompanied by the acquisition of lactase activity in the small intestines of the suckling neonates [1-5]. However, we have found that some carnivorous eutherian species are exceptional in this regard, given the fact that in their milk, oligosaccharides predominate over lactose [1-4, 140].

Robert Jenness, who comprehensively studied milk components in many mammalian species, reported from his paper chromatography data (1964) that in the milk of bears

oligosaccharides predominate over lactose [75]. Urashima et al. studied the chemical structures of oligosaccharides isolated from the milks of brown bear, Japanese black bear, and polar bear; the results were published in 1997-2003 [141-144]. Fig. 14 shows the BioGel P-2 gel filtration profiles of the carbohydrate fractions separated from the milk of a Japanese black bear during three lactation periods. The peak designated as TKM-6 was due to lactose while TKM-1-TKM-5 corresponded to the milk oligosaccharides, showing that lactose was only a minor milk saccharide [142]; the same observation was also applicable to brown and polar bears [141,143]. Urashima et al. were able to obtain samples of the milk or colostra of various carnivorous species including dog [145], striped skunk [146], mink [147], raccoon [148], white-nose coati [149], American black bear [150], giant panda [151], hooded seal [152], harbour seal [153], bearded seal [154], spotted hyena [155], African lion [156], clouded leopard [156] and cheetah [150] and to characterize their milk oligosaccharides together with an estimation of the ratio of milk oligosaccharides to lactose. Neutral oligosaccharides were isolated and purified using gel filtration of the carbohydrates and HPLC with a graphite carbon column, while acidic oligosaccharides were purified using normal-phase HPLC with an Amide-80 column, after which the oligosaccharides were characterized using ^{1}H-NMR and MALDI-TOFMS.

The structures of the representative neutral oligosaccharides characterized in these milks/colostra, are shown in Table 2 [140]. The unique feature is that the A antigen [GalNAcα1-3(Fucα1-2)Gal], B antigen [Galα1-3(Fucα1-2)Gal], H antigen（Fucα1-2Gal, and α-Gal epitope [Galα1-3Galβ1-4Glc(NAc)] are extensively found at the non-reducing ends of the milk oligosaccharides in these species, although the distribution of these oligosaccharide units differs depending on the species. In human milk, these types of oligosaccharides, other than those containing the H antigen, occur only at trace concentrations or are not present [79, 80, 84]. Human milk does not contain oligosaccharides with the α-Gal epitope, probably because α1-3-galactosyltransferase, which transfers Gal from UDP-Gal to OH-3 of Gal of lactose or LacNAc, was lost in humans and apes after their divergence from the Old World monkeys [157]. Among these species, only the milk oligosaccharides of *Ursidae* species, including Japanese black bear, polar bear, brown bear and American black bear, contain the Lewis x [Galβ1-4(Fucα1-3)Glc(NAc)] unit [141-144]. Among *Ursidae*, the distribution of non-reducing ABH antigens differs; some oligosaccharides of polar bear milk contain the A antigen [143], whereas those of Japanese black bear [142,144], American black bear [150], and polar bear have the B antigen [143], while those of brown bear [141] and American black bear [150] have the H antigen. Among the oligosaccharides shown in Table 2, 2'-FL has been identified in the milks of many species other than the giant panda [151] and the clouded leopard [156], while oligosaccharides containing the H type 2 units (Fucα1-2Galβ1-4GlcNAc)

were found only in the milks of the raccoon [148], white-nose coati [149], hooded seal [152], and harbour seal [153]. Isoglobotriose (Galα1-3Galβ1-4Glc) has been found in the milks of carnivorous mammals other than those of dogs, raccoons, hooded seals, harbour seals, African lions and clouded leopards [140], whereas Galili pentasaccharide (Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc), a saccharide containing a non-reducing α-Gal epitope, has been detected only in the milks of the striped skunk [146], white-nose coati [149], and polar bear [143]. Galα1-3Galβ1-4(Fucα1-3)Glc or Galα1-3Galβ1-3(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc, which have both the α-Gal epitope and Lewis x, have been identified only in the milks of four bear species (Japanese black bear, American black bear, polar bear and brown bear) [141-144,150]. The A tetrasaccharide [GalNAcα1-3(Fucα1-2)Galβ1-4Glc] has been identified in the milks of dogs [158], striped skunks [146], polar bears [143], African lions [156], and clouded leopards [156], whereas the B tetrasaccharide [Galα1-3(Fucα1-2)Galβ1-4Glc] was found in the milks of Japanese black bear [142], American black bear, [150] polar bear [143] and spotted hyena [155]. Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc, which contains the Lewis y [Fucα1-2Galβ1-4(Fucα1-3)GlcNAc] unit, was detected only in milks of the brown bear [141] and American black bear [150]. The milk/colostrum of some species have oligosaccharides containing lacto-*N*-neotetraose or lacto-*N*-neohexaose as core units other than the lactose core. Type □ oligosaccharides have not been detected in the milks/colostra of any of these species.

Regarding sialic acid in acidic oligosaccharides, the milks of most species contain only Neu5Ac and no Neu5Gc; these include dogs, minks, striped skunks, raccoons, Japanese black bears, American black bear, giant pandas, Harbour seals, spotted hyenas, African lions, and clouded leopards [144,146,147,148,150,151,153,155,158], whereas those of African lions and clouded leopards contained only Neu5Gc, or else Neu5Gc predominated over Neu5Ac [156]. In the milks of minks [147], striped skunks [146], Japanese black bears [144], American black bears [150], and harbour seals [153], Neu5Ac is attached to a lacto-*N*-neohexaose core unit via the α2-6 linkage, while in the milk of raccoons [148], Neu5Ac is attached to the core unit via α2-6 or α2-3 linkages. Lactose -O-3'-sulphate was identified only in the milks of dogs [145,158] and cheetahs [150].

In Fig. 15 the structures of the high-molecular-weight oligosaccharides are compared between raccoon and tiger quoll, a marsupial. The oligosaccharides in raccoon milk are elongated from lacto-*N*-neohexaose functioning as a mother core structure [148], while those in tiger quoll milk are elongated vertically to digalactosyllactose functioning as the mother core, by the attachment of two units of LacNAc [59]. These findings illustrate the heterogeneity of the high-molecular milk oligosaccharides between eutherians and marsupials.

Fig. 16 shows pie charts of the ratios of milk oligosaccharides to lactose, estimated from the peak areas in the gel filtration profiles, in the milks of several eutherian carnivorous species, compared to those in the Artiodactyla species as a reference [140]. It shows that milk oligosaccharides predominate over lactose in the milks of Caniformia (other than dogs), including bears, seals, skunks, raccoons, and minks. The concentrations of oligosaccharides and lactose are rather similar each other in the milks of Feliformia including hyenas, cheetahs, clouded leopards and lions, but the ratios in both Caniformia and Feliformia species are higher than those in the Artiodactyla. In the milk of house dogs, lactose is an exception among Caniformia, being dominant over oligosaccharides; this may be due to a decrease in their milk oligosaccharides as a result of prolonged breeding by humans.

The high ratio of milk oligosaccharides to lactose in the milk of carnivorous species, especially in Caniformia, may be caused by a decrease in the expression level of α-lactalbumin in the mammary cells of lactating females, causing a reduction in the rate of lactose biosynthesis relative to that of the biosynthesis of milk oligosaccharides [1-4,140]. What could be the physiological reason for a retardation of α-lactalbumin expression? In bears, suckling neonates depend on fat rather than lactose as their chief energy source [159,160,161]. Female bears give birth while fasting during the denning season [159, 160] and It is advantageous for the mother to incorporate high concentrations of fat in her milk, derived from her subcutaneous fat, rather than lactose; this enables her to maintain a constant blood glucose level, thus preventing damage to her brain [159, 160]. In the case of seals, which are marine mammals, neonates must rapidly accumulate subcutaneous fat to maintain their body temperature [161]. In addition, as the lactation period is very short, a high concentration of fat in their milk is advantageous to the rapid growth of their young [161]. Given that seal neonates depend for growth on milk fat as their major energy source, the significance of lactose in seal milk could have been lost [1-3]. Presumably the low concentration of lactose in seal milk was caused by a low expression of α-lactalbumin in the mammary glands; it can also be speculated that this might have been caused by a decrease in glucose, a substrate for lactose synthetase, in the mammary cells, possibly due to a retardation of the transport of glucose from the circulation. Despite a decrease in the expression level of α-lactalbumin in the mammary cells, the expression levels of glycosyltransferases would have been retained; thus, the ratio of milk oligosaccharides to lactose in milk increased in these species.

Raccoons and other carnivorous species are not marine mammals and do not have a denning period. Nevertheless, why has the ratio of milk oligosaccharides to lactose increased in their milks? The reasons for this remain to be explored in future studies.

The milks of whales and dolphins and other marine mammals, whose ancestors are

Artiodactyla species, contain low concentrations of lactose as the dominant saccharide as well as even lower concentrations of milk oligosaccharides, in the presence of a high concentration of fat [162,163,164].

8. Structural diversity of milk oligosaccharides among mammalian species, mainly in terms of bovine milk oligosaccharides

In the milk or colostra of many domestic farm animals, lactose is the dominant saccharide, while milk oligosaccharides are present at trace levels. As it had previously been difficult to isolate available amounts of these oligosaccharides for structural characterization, there had been only few studies, including studies on the structural characteristics of the oligosaccharides of cow milk, an important food source. The chemical structures of some major bovine sialyl oligosaccharides were characterized by Kuhn and Gauhe in 1965 [165] and Schneier and Rafelson in 1966 [166], while a few neutral oligosaccharides were characterized by Tadao Saoto in 1984 [167] and 1987 [168], and then by Urashima in 1991 [62]. Although bovine colostrum collected immediately post partum contains approximately 1 g/L of sialyl oligosaccharides, this level rapidly decreases after 48 h post partum and reaches trace levels in mature milk [169]. The concentrations of neutral bovine oligosaccharides are low in both the colostrum and in mature milk [170]. These low concentrations were limiting factors in the studies on their chemical structures. Urashima et al. used several liters of bovine colostrum to purify neutral oligosaccharides using paper chromatography, a time-consuming procedure [62]. The purification and characterization techniques for milk oligosaccharides have since greatly improved, and one can determine their structures using only 1 mL of the sample and a short analysis period. As a result, much structural information about milk oligosaccharides is now available, illustrating the diversity of milk oligosaccharides among mammalian milks/colostra. In this section, the historical aspects of the characterization of milk oligosaccharides are described mainly with respect to bovine samples and their structural differences from HMOs are discussed. A few reviews have been published for the milk oligosaccharides of cows [171, 172] and goats [173]

Among bovine colostrum sialyl oligosaccharides, the following structures were characterized by Kuhn and Gauhe in 1965 and Schnaier and Rafelson in 1966; Neu5Acα2-3Gal, Neu5Acα2-3Galβ1-4Glc (3'-SL), Neu5Acα2-6Galβ1-4Glc (6'-SL), Nau5Gcα2-3Galβ1-4Glc, Nau5Gcα2-6Galβ1-4Glc, Nau5Acα2-6Galβ1-4GlcNAc(6'-SLN), Nau5Gcα2-6Galβ1-4GlcNAc, and Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc (DSL) [165,166]. Among the neutral oligosaccharides Saito et al. published the structures of Galβ1-4GlcNAc (LacNAc), GalNAcβ1-4Glc, Galβ1-3Galβ1-4Glc (3'-GL), Galβ1-6Galβ1-4Glc (6'-GL), and Galβ1-

4(Fucα1-3)GlcNAc in 1984 [167] and 1987 [168], while Urashima et al. in 1991 characterized the structures of Galα1-3Galβ1-4Glc (isoglobotriose), GalNAcα1-3Galβ1-4Glc, and lacto-*N*-novopentaose □ [62]; these were determined using ^{13}C-NMR spectroscopy and classical analytical methods of monosaccharide analysis and determination of the partially methylated alditol acetates prepared from the purified oligosaccharides using gas chromatography–mass spectrometry.

Structural study techniques have recently been improved. In 2008 Tao et al. extracted the oligosaccharides from bovine colostrum (0.5 mL), and isolated and determined their structures using HPLC with a graphite carbon column after reduction of the saccharides [174]. They then compared the relative retention times and mass fragmentation patterns with standard saccharides after nano electron spray and ion cyclotron resonance mass spectrometry. In 2011 and 2014, Marino et al. and Albrecht et al., respectively, isolated oligosaccharides by fractionation using weak anion exchange chromatography and then by HPLC with a hydrophilic interaction column (HILIC-HPLC) after labeling with 2-aminobenzamide [28, 63]. They then determined the structures using offline negative-ion mode mass spectrometry MS/MS technique in combination with sequential exoglycosidase digestions. In 2020, Africano-Remoroza et al. characterized bovine milk oligosaccharides (BMOs) using mass spectroscopy coupled with HILIC-HPLC and correlation to the MS/MS data publicly available for known structures [60]. Structural characterizations have been performed for the milk oligosaccharides of goats, sheep, water buffaloes, camels, horses, and pigs. As the structural studies were mainly performed using MS/MS base techniques, we suspect that the determinations of the linkage positions of fucose or sialic acid to core structures, and the differentiation between type 1 and 2 structures, may sometimes not be reliable when carried out only by these techniques. When the determinations are difficult, a combination of exoglycosidase digestion and HPLC-MS techniques are available for precise characterization, as in the studies by Marino et al. [28] and Albrecht et al. [63].

The possible biosynthetic pathways for BMO core structures are shown in Fig. 17 [171], in which it is noteworthy that some of the intermediate oligosaccharides have not so far been detected in HMOs. The *iso* lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-6Galβ1-4Glc), which would be synthesized from *iso* lacto-*N*-triose (GlcNAcβ1-6Galβ1-4Glc) as a precursor, was first identified in horse colostrum in 1991 by Urashima et al. [175] and detected in the milks/colostra of goats, sheep, pigs, and camels [60,63]. The activity of β1-6-N-acetylglucosaminyltransferase (IGnT), which transfers GlcNAc from UDP-GlcNAc to the OH-6 position of Gal of lactose to synthesize *iso* lacto-*N*-triose, is assumed to be present in lactating mammary glands of these animals, but not in humans. The major oligosaccharide, lacto-*N*-novopentaose □, must presumably be synthesized from lacto-*N*-novotetraose

[Galβ1-3(GlcNAcβ1-6)Galβ1-4Glc]. This would be synthesized from 3'-GL by another IGnT which transfers GlcNAc to OH-6 of the penultimate Gal of 3'-GL. The activity of this type of IGnT has been identified in homogenates of the lactating mammary gland of the tammar wallaby, a marsupial [71]. Because trace levels of {Neu5Acα2-3Galβ1-3[Galβ1-4(Fucα1-3)GlcNAcβ1-6]Galβ1-4Glc}, the sialyl and fucosyl derivative of lacto-*N*-novopentaos □, have been found in human milk [67], this type of biosynthesis is speculative but would be a minor route for the biosynthesis of HMOs. Whether or not lacto-*N*-novopentaose □ is the major oligosaccharide in milk/colostrum may depend on the concentration of 3'-GL within the lactating mammary cells. β3GalT, which transfers Gal to OH-3 of Gal of lactose, and iGnT, which transfers GlcNAc to the same position, have specificity for the same substrate, implying competition between the activities of these two transferases. Whether either of LN*n*T, LNT, LNH or LN*n*H predominates over lacto-*N*-novopentaose □ in the milk or vice versa, would depend on the strength of the activity of iGnT or of β3GalT in the lactating mammary glands of a species.

GalNAcβ1-4Galβ1-4Glc (Asialo-GM_2 trisaccharide) and Galβ1-3GalNAcβ1-4Galβ1-4Glc (Asialo GM_1 tetrasaccharide) are similar to the carbohydrate moieties of ganglio-type glycolipids, Asialo-GM_2 and Asialo GM_1. These kinds of saccharides have not so far been detected among HMOs [79,80], possibly due to the absence of the activity of β1-4N-acetylgalactosaminyltransferase (β4GalNAcT), which synthesizes the Asialo-GM_2 trisaccharide from lactose. Another ganglio-type milk oligosaccharide, GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc (GM_2 tetrasaccharide) has been found in the milks of bottlenose dolphin [162], rhesus macaque [176] and giraffe [61].

Although bovine colostrum contains isoglobotriose [62], GalNAcα1-3Galβ1-4Glc [62], GalNAcβ1-4Glc [28,167], LacNAc [168] and its sialyl derivatives [165], and Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc (DSL) [165], these have not been found among HMOs [79,80]. Galα1-4Galβ1-4Glc (globotriose), the only globo-type oligosaccharide, has been identified in the milk of bottlenose dolphin [162] and sitatunga [61], an Artiodactyl, but has not been detected in bovine milk/colostrum or in human milk. Neu5Gc containing oligosaccharides have been identified in the colostra of domestic farm animals such as cows [28,63,165], goats [63,177], and sheep [63,178,179], but these kinds of oligosaccharides have not so far been isolated and characterized from human milk [79,80]. As aforementioned, in human milk type 1 oligosaccharides predominate over type 2, in contrast to the milk/colostrum of the domestic farm animals studied to date, which do not contain type 2 or contain it only at low concentrations.

The development of structural study methods for milk oligosaccharides enabled their characterization even when present at very low concentrations. The resulting studies

demonstrated that there is a large structural diversity of milk oligosaccharides among mammalian species.

The oligosaccharide concentrations in bovine milk samples rapidly decrease after 48 h postpartum and reach trace levels in mature milk [169]. In ruminant mammals, the microflora is constructed within the first stomach; this has a significant effect on digestion of the feed, and the colonic microflora may not have much significance. Therefore, bovine milk oligosaccharides may not be so important for the suckling bovine neonates and, by negative selection, the concentrations of oligosaccharides and the ratio of milk oligosaccharides to lactose have become low in the milks/colostra of ruminants, as illustrated in Fig. 16 [140].
Are milk oligosaccharides significant for animals such as horses and elephants, whose feed is digested in the cecum? Can milk oligosaccharides be digested by the cecum microflora of such animals? Are milk oligosaccharides utilized by intestinal bacteria, other than bifidobacteria, in the microflora of non-human mammals? These are among many questions about milk oligosaccharides that remain to be answered. Previous observations suggest that the milks of elephants contain relatively high concentrations as well as many varieties of oligosaccharides; this animal may therefore be a suitable model for studies concerning the utilization of milk oligosaccharides by microbiota in the cecum [180,181,182].

9. Conclusion

During the evolution of mammals from synapsids, the acquisition of α-lactalbumin from lysozyme resulted in the appearance of lactose and of oligosaccharides in milk. It is hypothesized that oligosaccharides had initially predominated over lactose in the milk or milk-like secretions of female primitive mammals. This feature was conserved in living monotremes and marsupials, in which milk oligosaccharides function mainly as an energy source for the neonates. It has also been proposed that in suckling macropod marsupials and in monotremes, the milk oligosaccharides and lactose are transferred into the epithelial cells of the small intestine via pinocytosis or endocytosis, whereupon they are digested by intracellular lysosomal enzymes. This mechanism of absorption and digestion would be relatively slow when compared with the eutherian mechanism for lactose, which could explain the well-known phenomenon of lactose intolerance in pouch-young kangaroos, wallabies and some other marsupials. In eutherians, as a result of a hypothetical increase in the expression of α-lactalbumin, lactose became dominant over a lower concentration of milk oligosaccharides. The accompanying acquisition of lactase in the microvilli of the small intestinal epithelial cells enabled lactose to become a readily available energy source for the eutherian suckling young. The biological significance of eutherian milk oligosaccharides may

differ between species, but in human infants they are significant in stimulating the growth of colonic bifidobacteria and it is also likely that they inhibit infection by pathogenic bacteria and viruses, modulate immunity, strengthens the colonic barrier function, and act as nerve development factors. Further studies should aim to explore whether milk oligosaccharides stimulate the growth of colonic microorganisms such as bifidobacteria, and have other effects, in non-human mammals.

Heterogeneity and diversity of milk oligosaccharide structures are found in most of the mammals so far studied. It has been found that the ratio of milk oligosaccharides to lactose in milk varies, depending on the species. These variations are possibly due to differences between them in the physiological significance of their milk oligosaccharides.

Although most eutherian milks contain lactose as the dominant saccharide in addition to a low concentration of oligosaccharides, milk oligosaccharides predominate over lactose in milk/colostrum of the Carnivora, especially in Caniformia species. For bears and seals, suckling neonates depend on milk fat, but not on carbohydrates, as a major energy source. During evolution the lactose concentration in these milks would presumably have decreased due to low expression of α-lactalbumin, while the milk oligosaccharides remained because of the presence of several glycosyltransferase activities in the mammary glands. However, the physiological basis for the high ratio of milk oligosaccharides to lactose in other Caniformia species, including raccoons, still remains unknown. Similarly, the variations in the ratio of milk oligosaccharides to lactose in the milk of mammals in general are difficult to explain but are likely related to differences in their living strategies. However, detailed studies on milk oligosaccharides have so far been limited to certain species.

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Conflict of interests

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Tables legend

Table 1 The 20 core structures of human milk oligosaccharides. Adapt from Table 2 of ref. [80] with permission.

Table 2 Comparison of neutral milk oligosaccharides among some Carnivora species. Adapt from Table 15.1 of ref. [140] with permission.

Figures legend

Fig. 1 Amino acid sequences of platypus and echidna α-lactalbumin, and bovine lysozymes. Numbering is done for the amino acid sequence of the lysozyme. Adapt from Fig.

1 of ref. [2] with permission.

Fig. 2 The hypothetical evolution from lysozyme to α-lactalbumin. Adapt from Fig. 1 of ref. [3] with permission.

Fig. 3 The cover page of Science vol. 180, 1973. ref. [37] with permission.

Fig. 4 Gel filtration of milk carbohydrates of the echidna (a) and the platypus (b) on Sephadex G-15. Fractions were analyzed for hexose, fucose, and sialic acids; Ve is the elution volume and Vo is the void volume. These figures are reproduced from Fig. 1 of ref. [37] with permission. Fractions were analyzed for hexose (●), sialic acid (X), and fucose (○). Arrows show the Ve/Vo values of the oligo- and monosaccharides used for calibration of the column; G4, maltotetraose; G3, maltotriose; G2, maltose; G, glucose; SL, sialyllactose; DFL, difucosyllactose; FL, fucosyllactose; L, lactose; Gal, galactose; and F, fucose.

Fig. 5 The structures of neutral and acidic milk oligosaccharides of the platypus. CFG symbols are used to express individual monosaccharides and different colors, whereas different glycosidic linkages are shown by different bond angles in a clockwise format; *i.e.*, 1-2 linkage (6:00 O'clock), 1-3 (7:30), 1-4 (9:00), and 1-6 (10:30). On the other hand, α and β anomers are represented by *thin* and *thick* lines, respectively.

Fig. 6 The absorption and digestion of milk oligosaccharides by the suckling neonates of monotremes and marsupials (A) and those of lactose by the neonates of eutherians (B).

(A) The milk oligosaccharides enter the small intestinal cells via pinocytosis or endocytosis and are transferred to lysosomes in which they are hydrolyzed to monosaccharides by several acid glycosidases.

(B) The predominant saccharide, lactose, is split into glucose and galactose by an intestinal lactase (neutral β-galactosidase) which is located in the membrane of the microvilli of the small intestinal brush border, and these monosaccharides are transported into the enterocytes via specific mechanisms.

Fig. 7 Gel filtration of the carbohydrate of tammar wallby milk collected at (a) 5 weeks, (b) 26 weeks and (c) 32 weeks. These figures are reproduced from Fig. 3 of ref. [49]. Fractions were analyzed for hexose (●), sialic acid (○), and N-acetylhexosamine (□).

Fig. 8 The major linear series of neutral and acidic milk oligosaccharides of tammar

wallaby and red kangaroo, respectively.

Fig. 9 The branched oligosaccharides separated from the milks of tammar wallaby, red kangaroo, brushtail possum or eastern quoll.

Fig. 10 The hypothetical biosynthetic pathway of the neutral milk oligosaccharides found in the eastern quoll milk. Adapt from Fig. 6 of ref. [58] with permission.

Fig. 11 The hypothetical biosynthetic pathway of 19 core structures of human milk oligosaccharides, excepting that of lacto-*N*-novopentaose □. Adapt from Fig. 1 of ref. [80] with permission.

Fig. 12. HMO utilization pathways in *B. bifidum* (A) and *B. longum* subsp. *infantis* (B). (A) The mono- and disaccharides highlighted in orange are extracellularly produced by *B. bifidum* and are partly shared among gut microbes, especially within bifidobacteria (ref. [108]). (B) *B. longum* subsp. *infantis* has been reported to import almost all HMOs, but the transporters responsible for the uptake of several HMOs, indicated by asterisks, have not been identified. (A and B) The transporters for monosaccharides and lactose are not shown. This figure is adapted from ref. [105] with modifications (Licensed under CC BY; http://creativecommons.org/licenses/by/4.0/).

Fig. 13. Prevalence of HMO assimilation genes in *Bifidobacterium* genomes. The prevalence is indicated as the percent occurrence of each homolog gene in the genomes of *Bifidobacterium* species. The value in parenthesis indicates the number of the genomes examined. The occurrence of the homolog genes (identity ≥70 %; query coverage ≥60 %; e value $<1\times10^{-50}$) was examined by tblastn analysis as described previously (ref. [105]). The data presented are an update of ref. [105] (Licensed under CC BY; http://creativecommons.org/licenses/by/4.0/).

Fig. 14 Profiles of milk carbohydrate of Japanese black bear at three lactation periods by gel filtration on BioGelP-2 (2.5 ☒ 100 cm). The solid line shows the detection for hexose by phenol – sulfuric acid method at 490 nm, while the line with the white circle shows that for sialic acid by periodate – resorcinol method by 630 nm. Adapt from Fig. 1 of ref. [142] with permission.

Fig. 15 The high-molecular-weight oligosaccharides, isolated from the milks of (a)

raccoon, an euthrians and (b) tiger quoll, a marsupial. Adapt from Fig. 6 of ref [80] with permission.

Fig. 16 The ratio of milk oligosaccharides to lactose in milks among the Carnivora species and the Artiodactyla species. Adapt from Fig. 15.4 of ref. [140] with permission. (The illustration was done by MAKIKO GOTO)

Fig. 17 The hypothetical biosynthetic pathway of the neutral oligosaccharides in bovine milk/colostrum. Adapt from Fig. 1 of ref. [171] with permission.