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Published in:
Journal of Functional Foods

DOI:
10.1016/j.jff.2020.104176

Publication date:
2020

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Al-Khafaji, A. H., Jepsen, S. D., Christensen, K. R., & Vigsnæs, L. K. (2020). The potential of human milk oligosaccharides to impact the microbiota-gut-brain axis through modulation of the gut microbiota. *Journal of Functional Foods*, *74*, [104176]. https://doi.org/10.1016/j.jff.2020.104176

Download date: 09. okt.. 2020

Journal of Functional Foods 74 (2020) 104176

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

The potential of human milk oligosaccharides to impact the microbiota-gut-brain axis through modulation of the gut microbiota

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ARTICLE INFO

Keywords:
Human milk oligosaccharides
Microbiota
Human gut
Microbiota-gut-brain axis
Brain disorders
Neuroactive molecules

ABSTRACT

Human milk oligosaccharides (HMOs) are the first prebiotics humans meet in life. HMOs provide many benefits to infants including impact on gut bacteria, promotion of postnatal brain development, and modulation of the immune system.

A complex bidirectional communication pathway called the microbiota-gut-brain axis links gut homeostasis and microbial composition with the central nervous system (CNS). Several mechanisms regulated by the gut microbiota are known to affect this axis. Dynamics in microbiota composition changes the metabolite profile in the gut, including neuroactive biomolecules that influence brain function and health. The gut microbiota also has the potential to affect and interact with the neurologic, immunologic and endocrine pathways of the microbiota-gut-brain axis.

In this review, we discuss the potential role of HMOs in microbiota-gut-brain axis and CNS disorders, by describing the role of the gut microbiota in relation to brain health and the importance of dietary interventions in manipulating the microbiota-gut-brain axis.

1. Introduction

1.1. Microbiota-gut-brain-axis

The gut-brain axis refers to the important bidirectional link between the gastrointestinal tract and the central nervous system (CNS). It involves direct and indirect pathways between cognitive and emotional centers in the brain with peripheral intestinal functions (for a thorough review see: (Cryan et al., 2019)). Accumulating evidence show the importance of the gut microbiota on the gut-brain axis, demonstrated in animals as well as humans (Bercik et al., 2011; Savignac et al., 2013; Schmidt et al., 2015). Therefore, the term "microbiota-gut-brain axis" is now widely used, as the role of the gut microbiota is not limited to the intestines. A disruption or alteration of the gut microbiota has been related to several health burdens and diseases inside and outside the gut (Minato et al., 2017; Vogt et al., 2017). The link between microbiota disruption and several conditions makes the gut microbiota a promising therapeutic target in treating intra- as well as extra-intestinal diseases, including CNS disorders and it is being greatly investigated in several animal disease models and intervention studies (Burokas et al., 2017; Grimaldi et al., 2018; Sampson et al., 2016). The gut microbiota can interact with the gut-brain axis in different ways, including through an endocrine, immunologic or neurologic way all discussed in the review by Cryan et al., from 2019: (Cryan et al., 2019). The gut microbiota is also highly involved in the production, secretion or regulation of neuroactive biomolecules that either directly or indirectly can impact brain

Abbreviations: AD, Alzheimer's Disease; ASD, Autism Spectrum Disorder; BBB, Blood-Brain Barrier; BDNF, Brain Derived Neurotrophic Factor; BMOs, Bovine Milk Oligosaccharides; CNS, Central Nervous System; DFL, DiFucosyllactose; FA, Ferulic Acid; FAE, Ferulic Acid Esterase; FOS, Fructooligosaccharides; Fuc, Fucose; GABA, Gamma-Aminobutyric Acid; Gal, Galactose; GF, Germ-Free; Glc, Glucose; GlcNAc, N-acetylglucosamine; GOS, Galactooligosaccharides; HMO, Human Milk Oligosaccharide; HMOs, Human Milk Oligosaccharides; IBS, Irritable Bowel Syndrome; IDO, Indoleamine 2,3-dioxygenase; IFN-γ, Interferon-Gamma; LNnT, Lacto-N-neotetraose; LTP, Long-term Potentiation; PD, Parkinson's Disease; ROS, Reactive Oxygen Species; SCFA, Short-Chain Fatty Acid; SCFAs, Short-Chain Fatty Acids; Sia, Sialic Acid; SL, Sialyllactose; 2′FL, 2′-Fucosyllactose; 3FL, 3-Fucosyllactose; 3′SL, 3′-Sialyllactose; 6′SL, 6′-Sialyllactose

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https://doi.org/10.1016/j.jff.2020.104176
Received 3 January 2020; Received in revised form 24 August 2020; Accepted 25 August 2020
Available online 23 September 2020
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health or function through the gut-brain axis (Barrett, Ross, O'Toole, Fitzgerald, & Stanton, 2012; Hata et al., 2017; Rudzki et al., 2019). This underlines the importance of the gut microbiota in brain health.

1.2. The gut microbiota and its functions

Humans contain approximately 150-fold more bacterial genes than human genes (Qin et al., 2010). A majority of the microorganisms colonize the gut with the colon holding the highest concentration of bacteria. The gut microbiota is a diverse and specialized ecosystem influenced by several factors. Evidence continues to mount, suggesting that the colonization of the infant gut begins prenatally, in utero (Dogra, Sakwinska, Soh, Ngom-Bru, Brück, Berger, & Holbrook, 2015; Hu et al., 2013). Subsequently, the mode of birth delivery (Madan et al., 2016), milk-feeding (Azad et al., 2013; Bezirtzoglou, Tsiotsias, & Welling, 2011) and weaning (Odamaki et al., 2016) impacts the bacterial colonization the first year of life together with genetics (Yatsunenko et al., 2012), environmental factors (Stewart et al., 2018), and medication incl. antibiotic usage (Bennet, Eriksson, & Nord, 2002). After weaning, the gut microbiota profile begins to be more similar to adults (Avershina et al., 2016). In adults, the gut microbiota contains a core community of permanent colonizers with some quantitative fluctuation induced by several introduced changes such as diet and medication (Arumugam et al., 2011; Rajilić-Stojanović, Heilig, Tims, Zoetendal, & de Vos, 2013). Some genera are more impacted by changes through age. As an example, bifidobacteria are the most abundant genus present in the infant gut (Stewart et al., 2018). During adulthood the abundance decreases considerably, and remains relatively stable, but further decreases in old age (Kato et al., 2017; Odamaki et al., 2016). Lower abundance of bifidoacteria has been found in a variety of diseases, suggesting a role of bifidobacteria in health (see the following review: (Arboleya, Watkins, Stanton, & Ross, 2016)).

The gut microbiota is constantly modulated by internal and external factors. Unfavorable alterations in the composition of the gut microbiota by factors such as diet, medication or stress can lead to a disruption in the symbiotic relationship between the host and associated gut microbiota leading to both external and internal diseases. This kind of unfavorable alteration in the gut microbiota is defined as dysbiosis. Contrary, when the gut microbiota can keep living in a mutualistic relationship with the host without compromising health status, it is defined as a healthy gut microbiota (Wilkins, Monga, & Miller, 2019). Several reviews have aimed to describe the exact composition of a dysbiotic and healthy gut microbiota. However, a lot of variations and changes can occur in the same individual influenced by e.g. age, diet and medical use (for reviews on this topic see: (Gagliardi et al., 2018; Rinninella et al., 2019)). Modulating the gut microbiota has been indicated to be a possible way to not only affect intestinal health but also affect the brain.

The effect of the gut microbiota on general health has been widely investigated the last decades, especially by the use of model systems, including *ex vivo, in vitro*, germ-free (GF) mice, and knock out models. Although translational differences exists between these simplified models and the complexity of a human, these models are highly relevant to investigate specific structures and mode of actions (see the following review for discussion of translation from animal models to humans in relation to gut microbiota: (Turner, 2018)). By the use of model systems the gut microbiota has shown to be important in vitamin synthesis (Gustafsson, 1959), metabolite production (Barrett et al., 2012; Hata et al., 2017), development of the immune system (Clarke et al., 2010; Maslowski et al., 2009; Wen et al., 2008), intestinal barrier homeostasis (Cani, Possemiers, et al., 2009), digestion of complex macromolecules and gut motility (Husebye, Hellström, Sundler, Chen, & Midtvedt, 2001). The gut microbiota has also demonstrated to be relevant in brain health, a relationship that has been thoroughly investigated by the use of GF animals. Wikoff et al., compared plasma samples from GF mice with conventional mice and observed differences in the presence of certain biomolecules (Wikoff et al., 2009). These biomolecules included the neurotransmitter serotonin, which had a 2.8-fold higher concentration in the plasma of conventional mice compared with GF mice (Wikoff et al., 2009). The precursor tryptophan, however, was higher in the GF animals. Several other studies have also indicated a strong influence of the gut microbiota and the serotonergic system (Clarke et al., 2013; Hata et al., 2017; Lukić, Getselter, Koren, & Elliott, 2019), and finally, studies in rats have shown a relationship between the gut microbiota and brain health and function (Luo et al., 2014; Savignac et al., 2013). Correlation-studies and interventions studies in humans substantiates what is seen in the model systems in relation to the link between gut microbiota and brain health, indicating a translational possibility.

1.3. Human milk oligosaccharides

Human breast milk, the first and primary nutrition source for the infant is rich in nutrients, vitamins and bioactive compounds that support development and growth of the infant. Human breast milk influences development of the gut microbiota, the immune system and affects metabolic pathways (for review see: (Hennet & Borsig, 2016)). Human breast milk contains several important biomolecules such as lactoferrin, immune components and human milk oligosaccharides (HMOs). Lactoferrin has important potential in modulating the gut microbiota, as seen when correlating the amount of fecal bifidobacteria and lactobacilli and the concentration of fecal lactoferrin (Mastromarino et al., 2014). Milk also holds a great amount of immune components like antibodies, cytokines and defensins, to help form the immune system of the infant. Another important microbiota modulator and the third most abundant solid bioactive compound in human breast milk, is HMOs (Tonon, Miranda, Abrão, de Morais, & Morais, 2019). The remaining part of this review will focus on HMOs and their possible effect on the microbiota-gut-brain axis.

Around 200 distinct structures of HMOs have been identified, all having a lactose group on their reducing end (Tonon et al., 2019) (for review see: (Hennet & Borsig, 2016)). HMOs consist of various combinations of five monosaccharides: galactose (Gal), glucose (Glc), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (Sia) that are linked via several glycosidic bonds see review by Bode (Bode, 2012). HMOs can be divided into three groups depending on their chemical composition: the first group is the neutral core HMOs that contains only Glc, Gal and GlcNAc. The second group is the neutral fucosylated HMOs made up of lactose or a neutral core backbone decorated with one or more Fuc units. The third group is the sialylated HMOs made up of lactose or a neutral core backbone decorated with one or more Sia units (Tonon et al., 2019). One example of each HMO-structure is shown in Fig. 1.

HMOs are resistant to enzyme degradation and acidic conditions during the passage through the gastrointestinal tract and reach the colon in an intact form, where only specific gut bacteria are able to utilize them. The utilization by gut bacteria are highly dependent on structure, and hence HMOs are used differently depending on species and strain. Most strains of *B. longum* subsp. *infantis* and *B. bifidum* are able to utilize several different structures of HMOs (Asakuma et al., 2011; Yu, Chen, & Newburg, 2013), whereas *B. breve* strains only grow on structures belonging to the neutral core group such as Lacto-*N*-tetraose (LoCascio et al., 2007). HMOs can however also indirectly affect the gut microbiota milieu in different way. HMOs have shown to inhibit binding and colonization of certain pathogens in the intestine *in vitro* (Weichert et al., 2013), interact with the epithelial wall and affect cells of the immune system (Zhang et al., 2019). These effects help to indirectly avoid dysbiosis in the intestines (see the thorough review by Bode: (Bode, 2012)).

A unique characteristic of HMOs is their high concentration and great structurally diversity in human milk. The concentration of HMOs

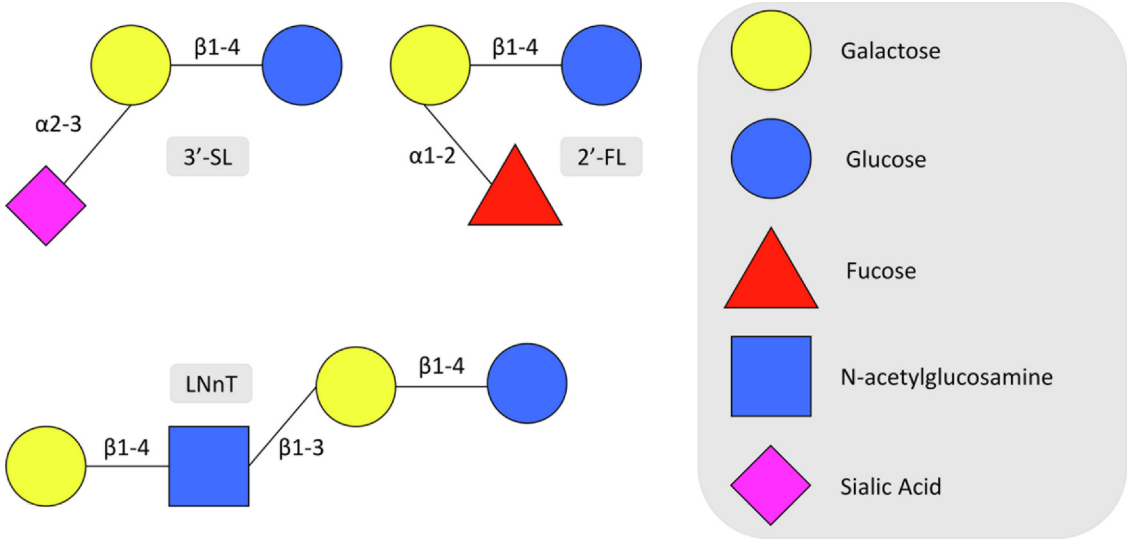

Fig. 1. Examples of the three different HMO-structures. 2′FL is an example of a neutral fucosylated HMO and the most abundant HMO in human milk, LNnT is among the neutral core HMO-structures and 3′SL is a sialylated HMO. Abbreviations used in this figure: HMO (human milk oligosaccharide); LNnT (Lacto-N-neotetraose); 2′FL (2′-Fucosyllactose); 3′SL (3′-Sialyllactose).

fluctuates during lactation with an estimate of 5–15 g/L present in human milk with colostrum having the highest concentration (Coppa et al., 1999; Tonon et al., 2019). The concentration and composition of the individual HMO-structures are affected both by genetic and non-genetic factors. Even though carbohydrate synthesis is not genetically encoded unlike nucleotides and proteins, HMO variability is strongly dependent on the activity of Secretor and Lewis genes in the mother (Kunz et al., 2017). No other animal produce the milk oligosaccharides to the same extent as humans and the concentration in most animal milk is very low with the range of 0.5–7 g/L with cows having the lowest and pigs the highest concentration (Tao et al., 2008; Difilippo et al., 2016). Although the individual structures of milk oligosaccharides are identical in human and animal milk, the sialylated oligosaccharides are highest in abundance in animal milk whereas the fucosylated oligosaccharides are highest in abundance in human milk (Coppa et al., 1999; Difilippo et al., 2016; Tao et al., 2008). It has been speculated to use bovine milk as a source of milk oligosaccharides for infant formula, however, as mentioned the concentration of milk oligosaccharides is very low in bovine milk with the majority of sialylated structures (Martín, Martín-Sosa, García-Pardo, & Hueso, 2001; Tao et al., 2008). Different groups have examined the effect of bovine milk oligosaccharides (BMOs). It was found in a study by Lane et al. that BMOs are able to affect the expression of cytokines and surface receptors on HT29 cells with a concentration of 4 g/L, which is higher than the physiological BMOs (Lane, O'Callaghan, Carrington, & Hickey, 2013). This shows that bovine milk holds oligosaccharides which have immunomodulatory functionality as HMOs. However, the concentration of oligosaccharides in bovine milk is not sufficient to induce these effects. Bovine milk still represent a potential source of milk oligosaccharides, and it has been speculated that whey permeate might be a source of the oligosaccharides, however it needs to be further investigated and optimized (Barile, Tao, Lebrilla, Coisson, Arlorio, & German, 2009). HMOs have until recently been reserved for breastfed infants. However, it is now possible to produce some of the most abundant HMO-structures. Some of the structures industrially produced includes Lacto-*N*-neotetraose ((LNnT), 2′-Fucosyllactose (2′FL), 3′-Sialyllactose (3′SL) and 6′-Sialyllactose (6′SL). Toxicology studies of these show no adverse, genotoxic or mutagenic effects, even at extremely high supraphysiological doses (Coulet, Phothirath, Allais, & Schilter, 2014; Coulet, Phothirath, Constable, Marsden, & Schilter, 2013; Monaco, Kim, Gurung, & Donovan, 2020; Parschat, Oehme, Leuschner, Jennewein, & Parkot, 2020; Phipps, Baldwin, Lynch, Flaxmer, et al., 2018; Phipps, Baldwin, Lynch, Stannard, et al., 2018; Phipps et al., 2019a, 2019b; Pitt et al., 2019). In clinical trials, 2′FL, LNnT and 3′SL have shown to be safe and well-tolerated in infants and adults (Elison et al., 2016; Kim, 2018; Puccio et al., 2017). Several products including infant formulas and digestive health products can now be found on the market with 2′FL and LNnT.

The most tested HMOs in humans are 2′FL and LNnT. Administration of 2′FL to infants have shown to lower the inflammatory cytokine profile in infants receiving infant formula compared to control group, mimicking the situation in breastfed infants (Goehring et al., 2016), and induce better cognitive development in infants in the first months (Berger, Plows, et al., 2020). A combination of 2′FL and LNnT have also shown to lower morbidity and decrease medical use in infants (Puccio et al., 2017). Supplementation of 2′FL and LNnT have also shown to modulate the gut microbiota specifically increasing the relative abundance of bifidobacteria in infants (Berger, Porta, et al., 2020) and adults (Elison et al., 2016) compared to controls. These clinical studies indicate that HMOs have a potential in general health. This review will discuss the potential role of the HMOs and CNS health by communicating through the microbiota-gut-brain axis.

2. How the microbiota can impact the brain

The gut microbiota can affect brain health in different ways e.g. through the neurologic, immunologic or endocrine pathways. Another way is by the regulation and production of numerous neuroactive biomolecules. These biomolecules are either produced directly during bacterial degradation of fibers or their biosynthesis is regulated by the gut microbiota. These neuroactive biomolecules function by stimulating the CNS and its processes. Among the neurotransmitters known to be influenced by the gut microbiota are ferulic acid (FA) (Szwajgier & Anna, 2010; Tomaro-Duchesneau et al., 2012), short chain fatty acids (SCFAs) (Baxter et al., 2019), gamma-aminobutyric acid (GABA) (Barrett et al., 2012), tryptophan (Gao et al., 2019; Rudzki et al., 2019) and serotonin (Hata et al., 2017; Yano et al., 2015).

FA, is a phenolic acid found in a vairety of plant structures including grains, seeds and leaves. FA exists freely but a fraction is linked to the plant cell by covalent bonds. Ferulic acid esterase (FAE) produced by bacteria can cleave the ester-bound FA making it available in the gut. FA has potential anti-inflammatory properties and inhibits generation of reactive oxygen species (ROS) (Chen et al., 2017; Maurya & Devasagayam, 2010). Increased ROS has been shown to play a role in

the development of neurodegenerative diseases see the following review: (Nissanka & Moraes, 2018). Moreover it has been shown by Yabe et al., that FA induces proliferation of adult neural stem cells and stimulates the expression of brain derived neurotrophic factor (BDNF) in the hippocampus of corticosterone-treated mice (Yabe et al., 2010). BDNF induces the differentiation and survival of cholinergic neurons in the basal forebrain (Ward & Hagg, 2000), as well as the secretion of acetylcholine. When BDNF is inhibited it results in cognition decline (Şahin et al., 2015). This indicates that promoting the right bacteria might increase FA-production and thereby increase brain health.

Similarly, SCFAs such as butyrate, acetate and propionate play important roles both intra- and extra-intestinally. SCFAs are produced by a range of different bacterial species. When bifidobacteria produce lactate and acetate, butyrate-producing bacteria can convert these metabolites to butyrate, a process known as cross-feeding (Schwab et al., 2017). SCFAs may act as endocrine signaling molecules that enter systemic circulation from the gut, cross the BBB through monocarboxylate transporters and reach the brain where these compounds can directly influence brain function, and SCFAs have also shown to improve BBB permeability in mice (Braniste, et al., 2014). SCFAs have also been shown to modulate synthesis and expression of nicotinic and GABA receptors (Nankova, Agarwal, MacFabe, & La Gamma, 2014), and can regulate the expression of tyrosine hydroxylase, an enzyme that plays an important role in the production of catecholamines, and is responsible for dopamine synthesis, degradation and transport (DeCastro et al., 2005). Furthermore, butyrate has been shown to stimulate the biosynthesis of BDNF in the frontal cortex in mice (Schroeder, Lin, Crusio, & Akbarian, 2007). Finally, GABA inhibits synaptic transmission in the brain, as it is the major inhibitory neurotransmitter of the CNS, and especially GABA produced from the gut microbiota has been suggested to have an impact on the gut-brain axis (Barrett et al., 2012; Strandwitz et al., 2019).

Tryptophan is an amino acid and a precursor to important neuroactive molecules. Tryptophan can either enter the kynurenine or the serotonin pathway. The serotonergic system has shown to be involved in several CNS disorders such as depression (Kelly & Borre, 2016; Owens & Nemeroff, 1994) and ASD (Boccuto et al., 2013). The serotonergic system is a highly relevant player in the microbiota-gut-brain axis, as a fraction of serotonin is synthesized from tryptophan by the enterochromaffin cells in the gut and some bacteria strains, like Lactobacillus, have also shown to directly synthesize serotonin from tryptophan *in vitro* (Özoğul, Kuley, Özoğul, & Özoğul, 2012). The kynurenine pathway on the other hand, leads to the production of kynurenine which can be further metabolized to 3-hydroxykynurenine and quinolinic acid, which have demonstrated to be neurotoxic. The gut microbiota has shown to impact the balance between the tryptophan degradation pathways and serotonin synthesis (Desbonnet, Garrett, Clarke, Bienenstock, & Dinan, 2008; Ge et al., 2017). As an example, an altered dysbiotic microbiota composition is often accompanied by an inflammatory state in the gut which can induce production of cytokines such as interferon-gamma (IFN-γ). Indoleamine 2,3-dioxygenase (IDO) is the rate limiting factor converting tryptophan to kynurenine in the kynurenine pathway, and is regulated by inflammation and IFN-γ (Jürgens, Hainz, Fuchs, Felzmann, & Heitger, 2009). Increased IFN-γ in a proinflammatory dysbiotic environment will alter the ratio of kynurenine:tryptophan. Kelly et al., showed that a fecal transfer from depressed patients to microbiota-depleted rats induced behavioral and physiological features characteristic of depression in the recipient animals including increased kynurenine:tryptophan metabolism (Kelly & Borre, 2016). This means a shift in the processing of tryptophan, limiting the amount of tryptophan entering the serotonergic pathway. In this way a dysbiotic microbiota composition can affect the serotonergic system contributing to different CNS disorders. This was illustrated in a study by Ge et al., where administration of antibiotics to mice decreased the diversity of the gut microbiota as well as serotonin levels (Ge et al., 2017). In contrast, a study by Desbonnet et al., showed that administration of a probiotic, *Bifidobacterium infantis*, increased plasma tryptophan and decreased pro-inflammatory cytokines in rats (Desbonnet et al., 2008). This illustrates a potential for the gut microbiota to not only affect the serotonergic system, but also regulating the immune system, another microbiota-gut-brain axis-pathway. Valles-Colomer et al., demonstrated that the microbiota phenotype is linked to mental health in humans (Valles-Colomer et al., 2019) and in a double blind, randomized, placebo controlled trial it was found that administration of probiotics *(Lactobacillus Plantarum 299v)*, lowered the amount of kynurenines and improved cognitive functions in patients with major depressive disorder significantly (Rudzki et al., 2019), underlining the importance of the gut microbiota in mental health.

The link between gut microbiota and brain health has been demonstrated in different studies. The gut microbiota is important for the development of an intact blood-brain barrier as shown in a study by Braniste et al., using GF mice. The GF mice had a more permeable BBB and lower expression of brain tight junction proteins compared to conventional mice. Interestingly, by inoculating GF mice with pathogen-free gut microbiota, the permeability of the BBB decreased (Braniste et al., 2014). Other studies in GF mice have demonstrated the influence of the gut microbiota on the expression of BDNF in the CNS (Arentsen, Raith, Qian, Forssberg, & Diaz Heijtz, 2015; Gareau et al., 2011). BDNF has shown to play an important role in the development and differentiation of neurons in murine models (Rauskolb et al., 2010; Strand et al., 2007), but is also believed to be important for learning and development of memory in humans, as showed by Egan et al., and Hariri et al., (Egan et al., 2003; Hariri et al., 2003). This is consistent with a study showing that decreased levels of hippocampal BNDF in mice is associated with anxiety- and depression-like behavior (Burokas et al., 2017).

Dysbiotic alterations in the composition of the gut microbiota, can therefore have a major impact on the gut-brain-axis associated conditions such as irritable bowel syndrome (IBS) (Kassinen et al., 2007), Alzheimer's disease (AD) (Vogt et al., 2017), Parkinson's disease (PD) (Minato et al., 2017; Sampson et al., 2016), autism spectrum disorder (ASD) (Liu et al., 2019) (Tomova et al., 2015), and major depression disorder (Jiang et al., 2015). These dysbiotic alterations include decreases in bifidobacteria, as well as a higher number of gram-negative bacteria and a decreased microbial diversity. In addition, gastrointestinal disturbances are among the early symptoms of neurodegenerative diseases, indicating that dysbiosis may participate in triggering diseases in the CNS (Forsyth et al., 2011). Thus, it is becoming clear that the gut microbiota is a critical component of the gut-brain axis, not only by regulating brain function, but also by regulating the physical development of the brain, as investigated by Heijtz et al., by the use of GF mice (Heijtz et al., 2011). Therefore, the gut microbiota might have great potential in the therapeutic field of CNS disorders, and modulation of the gut microbiota have been investigated as possible treatment options (Allen et al., 2016; Hadizadeh, Hamidi, & Salami, 2019; Pinto-Sanchez et al., 2017; Rudzki et al., 2019). In this section the focus has primarily been on the regulation of neuroactive biomolecules produced or regulated by gut microbiota, however, the microbiota-gut-brain axis includes several other pathways such as immunologic, neurologic and endocrine pathways. The neuroactive biomolecules introduced above also function by different mechanisms. Some work directly in the brain, whereas others regulates homeostasis and thereby indirectly affect brain health and function.

3. Modulation of the microbiota-gut-brain axis through the diet

As mentioned above, the gut microbiota composition and activity can be altered by diet and environmental factors (Tarr et al., 2015). In fact, diet is one of the most potent modulators of the gut microbiota. Wu et al., found that a Western diet dominated by proteins and animal fat induced a different gut microbiota profile compared with a diet high in fiber (Wu et al., 2011). This observation was also seen in a study by De

Filippo et al., where rural African children with a diet high in fiber content had a significantly different gut microbiota profile with a higher abundance of SCFA-producing bacteria compared with European children (De Filippo et al., 2010). In addition, a different SCFA-metabolite-profile have been observed in humans with inflammatory bowel disease compared to healthy controls (Zhuang et al., 2019). This includes decreased levels of total SCFA as well as the individual structures of acetate and valerate. Thus, modulating the gut microbiota through diet to improve the SCFA-profile could be a way to impact the microbiota-gut-brain axis and as a consequence improve health, as SCFAs also have other beneficial effects e.g. on the immune system (Haghikia et al., 2015). Sia is another compound obtained through diet essential for development and cognition (see review by Wang: (Wang, 2009)). Sia is a part of the gangliosides and the polysialic acid chains in the brain. Gangliosides are Sia-containing glycosphingolipids. These structures possess several important functions in relation to brain health and development. They have been indicated to be implicated in embryonic neural development and neuronal plasticity as well as potentiating long-term potentiation (LTP). The amount of ganglioside has been shown to be involved in different diseases and conditions (see review by Schengrund: (Schengrund, 2015)). Dietary supplemented with Sia has demonstrated increased learning and memory in piglets (Wang et al., 2007). This indicates a need for Sia in the brain, and dietary interventions are one way to obtain this. Biomolecules can be synthesized from undigested food products that reach the colon, which emphasize the importance of the diet in production of these essential molecules in managing brain health. Indigestible dietary fibers can impact the gut microbiota composition, in particular increasing saccharolytic bacteria, leading to the production of SCFAs, like butyrate. A majority of the produced butyrate is used by the colonocytes as an energy source (Donohoe et al., 2011). The rest enters the bloodstream and is either absorbed by the liver or enters systemic circulation. Wolever and Chiasson showed in a randomized, double-blind, placebo-controlled trial using subjects with impaired Glc-tolerance that treatment with acarbose (an anti-diabetic drug) caused an increase in acetate and butyrate concentrations in serum. This was argued to be caused by the effect of acarbose increasing the entry of indigestible dietary fibers into the colon, and hence stimulating saccharolytic bacteria (Wolever & Chiasson, 2000). The increase of circulating butyrate could have a direct function on the CNS. This has been shown in a pig model, where oral butyrate impacted brain metabolism and hippocampal neurogenesis (Val-Laillet et al., 2018). In addition, other studies have demonstrated that a diet high in fiber can influence the brain by providing it with essential nutrients for development, decreasing neuroinflammation, and supply of energy (Khan et al., 2015). In relation to this, another study observed a decrease in butyrate producing bacteria in patients with depression, demonstrating a link between gut, microbiota and brain health (Valles-Colomer et al., 2019).

New nutritional strategies to improve microbiota-gut-brain health are currently under investigation and are generating increasing interest. These strategies include the use of probiotics. A clinical study by Messaoudi et al., showed that the administration of probiotics (*Lactobacillus helveticus* and *Bifidobacterium longum*) alleviated psychological distress in rats and healthy volunteers (Messaoudi et al., 2011). In another trial, probiotic administration of *Bifidobacterium longum* reduced depression, but not anxiety scores in IBS patients. These improvements were associated with changes in brain activation patterns indicating that administration of *Bifidobacterium longum* reduced limbic reactivity (Pinto-Sanchez et al., 2017). Results as these have suggested a relevance for probiotics in brain health and function.

Also prebiotics have been thought to have a beneficial effect. Prebiotics is defined as: “A substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017). Some well-known prebiotics are oligosaccharides such as fructooligosaccharides (FOS) and galactooligosaccharides (GOS) or saccharide polymers such as inulin. Prebiotics have been shown to stimulate the growth of bifidobacteria and lactobacilli in the colon resulting in lower pH, due to the production of organic acids (Yu et al., 2013). In one study, FOS and GOS supplementation in rats influenced the composition of the gut microbiota and increased hippocampal BDNF expression, which was suggested to function through the production of gut hormones (Savignac et al., 2013). In a human trial, consumption of inulin caused an increase in gut microbiota fermentation, decreased appetite, improved postprandial Glc-responses and increased the concentrations of the two gut satiety hormones glucagon-like peptide 1 and peptide YY (Cani, Lecourt, et al., 2009). Both satiety hormones have been shown to be influenced by changes in the gut bacteria and particularly SCFAs by the use of knock-out animal models (Samuel et al., 2008; Tolhurst et al., 2012). As previously discussed, another available prebiotic is HMOs. HMOs have been shown to be essential for shaping the enteric microbiota of the infants (Berger et al., 2020; Marcobal et al., 2010). Also, fecal samples from breast-fed infants carry a more uniform microbiota population with a higher number of bifidobacteria compared with formula-fed infants that were not exposed to HMOs (Bezirtzoglou et al., 2011). HMOs have also shown to affect brain health and function, which is further discussed in Section 5.

4. HMOs and their impact on the gut microbiota and potential to affect the microbiota-gut-brain axis

HMOs have various biological functions, such as preventing the attachment of pathogens to epithelial cells (Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003) and modulating immune cell responses (Zhang et al., 2019) *in vitro*, as well as specifically modulating gut microbiota in infants as well as healthy and diseased adults (Berger et al., 2020; Elison et al., 2016; Iribarren et al., 2020) (for a review see: (Bode, 2012)). Yu et al., demonstrated the specific stimulation of gut bacteria by fucosylated HMOs (2′FL, 3FL and DFL) in an *in vitro* anaerobic culture system using infant fecal microbiota. By adding HMOs, the growth of bifidobacteria was stimulated with an increase in lactate production and a decrease in pH. In parallel, supplementation of FOS was investigated and the results showed that the production of organic acids was higher with HMO supplementation (Yu et al., 2013). In a clinical trial in infants, it was found that 2′FL and LNnT were safe, well tolerated and could shift the gut microbiota, particularly by increasing bifidobacteria, getting a phenotype closer to that observed in breastfed infants (Berger et al., 2020; Puccio et al., 2017). Interestingly, clinical trials using 2′FL and LNnT supplementation in healthy adults and IBS patients also showed modulation of the gut microbiota with increase in the abundance of bifidobacteria (Elison et al., 2016; Iribarren et al., 2020)

As mentioned, HMOs impact the composition of the gut microbiota, especially by inducing the growth of bifidobacteria (Elison et al., 2016). *Bifidobacterium* spp. are important members of the gut bacterial community, particularly due to their production of SCFAs, such as acetate and lactate. These can later serve as substrates for other bacteria that produce butyrate (Rivière, Gagnon, Weckx, Roy, & De Vuyst, 2015; Schwab et al., 2017). In CNS disorders like AD and ASD an altered microbiota composition has been associated with a particularly decreased level of *Bifidobacterium* (Vogt et al., 2017; Xu, Xu, Li, & Li, 2019). Manipulating the gut microbiota is recently being speculated as an alternative treatment for ASD (Grimaldi et al., 2018). Similar observations have been seen in patients with depression, holding a decreased amount of butyrate producing bacteria (Valles-Colomer et al., 2019). Furthermore, alterations in the gut microbiota profile of PD patients have also been observed and linked to reduced levels of fecal SCFAs. In a study investigating the gut microbiota composition of healthy individuals and PD patients, an increase in proinflammatory Proteobacteria species and a decrease in butyrate-producing bacteria of the genera *Roseburia* and *Faecalibacterium* was observed in PD patients (Keshavarzian et al., 2015). Hence, we speculate that increasing the amount of butyrate-producing bacteria and thereby the amount of

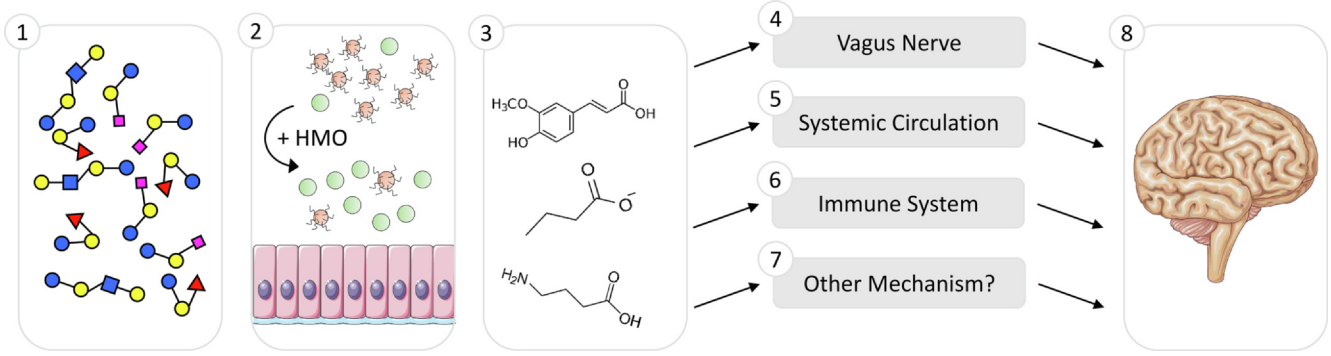

Fig. 2. The hypothetical impact of HMOs on the production and regulation of neuroactive molecules. HMOs are administered (1) and induce the growth of beneficial bacteria species, like *Bifidobacterium* spp. (2) which promote the production or activation of important biomolecules, like the SCFAs, GABA and FA (3). These molecules can either activate the vagus nerve (4), enter the systemic circulation (5), affect the immune system (6) or interact with the microbiota-gut-brain axis through other unknown mechanisms (7) and in that way impact brain functions (8). Abbreviations used in this figure: HMOs (human milk oligosaccharides); FA (ferulic acid); GABA (gamma-aminobutyric acid); SCFAs (short-chain fatty acids).

butyrate could have advantageous effects in PD patients. Since HMOs have been shown to stimulate the growth of bifidobacteria and by cross-feeding increase the production of butyrate, we speculate that this could contribute to attenuating the symptoms of PD patients. By inducing the production of SCFAs, HMOs may be a useful tool in long-term management of different CNS disorders.

GABA is important in the microbiota-gut-brain axis, and certain *Bifidobacterium* and *Lactobacillus* species including *L. brevis* and *B. adolescentis* contain genes responsible for the biosynthesis and transport of GABA (Siragusa et al., 2007; Barrett et al., 2012). These species convert monosodium glutamate into GABA in the gut by the enzyme glutamate decarboxylase (Komatsuzaki, Nakamura, Kimura, & Shima, 2008). GABA regulates the production of acetylcholine, dopamine, and serotonin, and hence could potentially be a target for CNS diseases like AD. Dysfunction of the GABAergic system have shown to contribute to the development of stress-related conditions, memory and cognitive impairments, as demonstrated in a murine model by Bravo et al., (Bravo et al., 2011). Interestingly, decreased levels of GABA in the frontal, temporal and parietal cortex have been observed in AD patients (Lowe et al., 1988). Therefore, a dysbiotic microbiota causing a reduction in GABA-producing bacteria species in the gut could result in reduced levels of GABA, and correlate with a decrease of GABA in the CNS. We hypothesize that HMOs could increase the production of GABA through stimulation of GABA-producing bacteria like the bifidobacteria in the gut and increase levels of GABA available to the CNS.

Several neurodegenerative diseases, including AD, PD and several others, are known to be related to increased ROS in the CNS, see the following review: (Nissanka & Moraes, 2018). FA is a potent antioxidant that neutralizes ROS (Ogiwara et al., 2002), and recovers mitochondrial membrane potential. It has also been shown in a sea urchin embryo model system that FA blocks genes related to apoptosis (Picone, Nuzzo, & Di Carlo, 2013). Some gut bacteria, including some *Bifidobacterium* spp., are capable of producing FAE (Kelly, O'Callaghan, Kinsella, & van Sinderen, 2018; Szwajgier & Anna, 2010), an enzyme involved in synthesizing FA (Tomaro-Duchesneau et al., 2012). By stimulating the beneficial FAE-producing bacteria, HMOs could indirectly participate in increasing the amount of FA and thereby neutralize the ROS accumulation present in different neurodegenerative diseases.

Managing the gut microbiota by HMOs, might also be one way to control the serotonergic system, known to be involved in numerous diseases, including CNS disorders. As stated earlier, tryptophan and its metabolite serotonin are strongly influenced by the gut microbiota. A dysbiotic environment in the gut may result in inflammation with an effect on the serotonergic system, IFN-γ and the ratio of kynurenine:-tryptophan. It has been shown in rats that bifidobacteria decrease IFN-γ and other inflammatory components, as well as alter the kynurenine:tryptophan ratio (Desbonnet et al., 2008). In another study in rats it was shown that depressed animals had a higher kynurenine:tryptophan ratio, and a decreased amount of different bacteria, including bifidobacteria (Kelly & Borre, 2016). It has also been shown that administering probiotics (*Lactobacillus helveticus* and *Bifidobacterium longum*) to patients with major depressive disorders have a potential in lowering the kynurenine:tryptophan ratio (Kazemi, Noorbala, Azam, Eskandari, & Djafarian, 2019). HMOs are known for their prebiotic effect and their ability to specifically stimulate bifidobacteria in the gut of children as well as adults (Elison et al., 2016; Lawson et al., 2019). A healthy gut bacteria composition might be useful in a variety of CNS disorders related to tryptophan and serotonin metabolism, and HMOs could play an important role in helping with this. HMOs and their effect on the microbiota-gut-brain axis is therefore highly relevant, as it exerts great potential in managing some CNS disorders. Production and regulation of neuroactive biomolecules is one way HMOs might affect the microbiota gut-brain axis, especially due to their increase in bifidobacteria. However, HMOs could also affect the microbiota gut-brain axis through neurologic, immunologic or endocrine pathways. Neuroactive molecules can also exert effect on the brain through different mechanisms. For an overview of this hypothesis see Fig. 2.

Even though a lot of experiments *in vitro* and *in vivo* and a lot of correlations studies suggest a great role for HMOs in modulation of the gut microbiota, clinical trials investigating this aspect is still at its infancy. A lot of studies are ongoing, and hopefully more studies will address this aspect in future. However, some clinical studies have already evaluated this effect and are listed in Table 1.

In one clinical study, Elison et al., demonstrated a modification of the adult gut microbiota after administration of 2′FL and LNnT. The study showed substantial increase in the abundance of Actinobacteria and *Bifidobacterium* and a reduction in the abundance of Firmicutes and Proteobacteria (Elison et al., 2016). A similar finding was seen in the study by Berger et al., that demonstrated an increase in bifidobacteria in infants (Berger et al., 2020). These studies were both conducted in healthy population. Another study has shown that 2′FL and LNnT could impact the gut microbiota in IBS patients increasing the abundance bifidobacteria (Iribarren et al., 2020). Even though the clinical trials regarding administration and microbial shift are limited, they all point to the same conclusion, backing up preclinical data and correlation-studies: HMOs are able to impact the gut microbiota in infants and adults, favoring a healthy gut microbiota and inhibiting harmful pathogens that might disrupt the healthy symbiotic relationship in the gut. More clinical studies are needed to address more HMO-structures, investigate the kinetics and different populations groups (e.g. sex, age and health status).

Table 1
Clinical studies evaluating the effect of HMOs on gut microbiota. Abbreviations used in the table: Glc (glucose); HMO (human milk oligosaccharide); HMOs (human milk oligosaccharides); IBS (irritable bowel syndrome); LNnT (Lacto-N-neotetraose); 2′FL (2′-Fucosyllactose).

Structures	Concentration + duration	Type of study	Investigation	N	Outcome	Reference
2′FL + LNnT	2′FL, LNnT or 2′FL + LNnT (2:1 mass ratio; mix) at 5, 10, or 20 g per day or 2 g of Glc (placebo) daily for 2 weeks.	Clinical trial, healthy adults	Microbial investigation	N = 100	HMO supplementation increased abundance of Actinobacteria and *Bifidobacterium* and reduced abundance of Firmicutes and Proteobacteria	(Elison et al., 2016)
2′FL + LNnT	5 g and 10 g doses of 4:1 mix of 2′FL and LNnT orplacebo (Glc) orally for 4 weeks + 4 weeks wash-out period.	Clinical trial, IBS patients	Microbial investigation	N = 60	HMOs induced the growth of the *Bifidobacterium* in patients with IBS without aggravating gastrointestinal symptoms.	(Iribarren et al., 2020)
2′FL + LNnT	Infant formula supplemented with 1.0 g/liter 2′FL and 0.5 g/liter LNnT. From < 14 days of age to 4 months of age only the test diet was administered. From 4 months, solid food was allowed as a supplement to test formula until 6 months of age. Then, all infants received the same follow-up formula without HMOs until 12 months of age.	Clinical trial, healthy infants	Microbial investigation	N = 175 Reference group: N = 38	At 3 months the HMOs shifted stool microbiota composition and diversity toward that of breastfed babies (the reference group).	(Berger, Porta, et al., 2020)

A lot of the above-mentioned mechanisms build on the impact HMOs have on the gut microbiota and focus on the production and regulation of neuroactive molecules. However, as will be illustrated in the next section, different pathways exist when linking the gut and its microbiota to the brain. Biomolecules will often have various functions impacting the brain both directly or indirectly and will be a part of more than one pathway.

5. HMOs and their effect in brain health and function

Already several decades ago it was found that breastfed infants showed better cognitive results compared to formula-fed infants. In 1990, Lucas et al., demonstrated that human milk promotes postnatal brain development in preterm infants. Lucas et al., compared the cognitive development of infants fed formula without HMOs to breastfed infants, showing improved cognitive capacities in preterm breastfed infants up to 18 months of age (Lucas et al., 1990). At the age of 7.5–8 years old the children who received their mother's milk were doing significantly better in intelligence tests compared to the formula fed (Lucas, Morley, Cole, Lister, & Leeson-Payne, 1992). Part of this effect could be due to HMOs. In addition, already in the early nineteens, several studies have examined the impact of HMOs either directly or indirectly on brain health and function. In 1994, Krug et al., showed that 2′FL increased LTP in rats (Krug, Wagner, Staak, & Smalla, 1994), and in 1996, Matthies et al., found that 2′FL increased population spike amplitude and field excitatory postsynaptic potential, and had positive effect on LTP in rat brains (Matthies, Staak, & Krug, 1996). Contrary, 3FL had no effect on LTP, indicating a structural importance of the HMOs in relation to brain-effect.

In recent years several other studies conducted in animals have investigated the effect of specific HMOs on the function of CNS. As previously mentioned, the most commonly used HMO is 2′FL. This HMO-structure have been tested in different experiments related to brain health and function. Vázquez et al., showed that dietary supplementation with 2′FL, improved learning and memory skills and impacted brain function in mice and rats (Vázquez et al., 2015). The administration of 2′FL was also linked to improvements in LTP, as seen in some of the earliest studies. In another study, it was shown that 2′FL affected brain function and cognition through the vagus nerve in male adult rats (Vazquez et al., 2016). This was investigated by vagotomy of the animals prior to operant conditioning and LTP measurements. Animals receiving 2′FL and sham surgery reached the selected criterion in the operant conditioned test in less sessions than the 2′FL-vagotomized, the control-vagotomized and the control-sham groups. The same was seen in regard to LTP. The 2′FL sham-operated group presented significantly larger LTP values than the other three groups (Vazquez et al., 2016). Interestingly, it was also shown that fucose did not have the same effect on LTP when administered orally. This suggests the necessity of 2′FL integrity to exert effects on CNS function (Vazquez et al., 2016), although the exact mechanism of how the HMOs can affect the microbiota-gut-brain axis is not yet understood. It was demonstrated in a metabolic study in mice by Kuntz et al., using labelled 2′FL that it does not reach the brain in its intact form. The majority of the 2′FL either applied intravenously or orally ended up in the gut, and was suggested to be modulated by the microbiota as differences were observed when comparing plasma levels of 2′FL in GF mice to wild type mice (Kuntz et al., 2019). This could suggest that the impact of 2′FL on the brain is depending on the gut microbiota. Still there is also the possibility that the signaling to the brain happens through another microbiota-gut-brain pathway e.g. the vagus nerve, as was demonstrated by Vazquez. In another study by Bravo et al., *Lactobacillus. rhamnosus* altered GABA-receptor expression in the brain and reduced stress-induced corticosterone and anxiety- and depression-related behavior in mice. These neurochemical and behavioral effects were not seen in vagotomized mice, also pointing to the vagus nerve as a relevant communication pathway between the gut bacteria and the brain (Bravo

et al., 2011).

2′FL has also demonstrated to have a stress-reversing effects on intestinal motility and propagating contractile clusters. This was investigated in two different studies (Bienenstock et al., 2013; Farhin et al., 2019). In addition, 2′FL has been shown to change the composition of cecal microbiota and metabolites in mice compared to low-fat diets and high fat diets, and modulated hyperphagic response to high-fat diets. In this study, there was a hint that the vagus nerve could be involved, however, the findings were not significant (Lee et al., 2020).

It was recently reported that 2′FL has beneficial effects in a murine stroke-model. 2′FL reduced neurotoxicity, apoptosis, and Ca++ influx in primary neuronal culture. Treatment with 2′FL before or after the induction of the stroke reduced brain infarction, motor deficits and inflammation and caused an increase in the expression of BDNF and glial cell-derived neurotrophic factor (Wu et al., 2020). These findings indicate that 2′FL might have a relevant role in brain health and function, but a lot remains to be investigated, including the mode of action and the involved microbiota-gut-brain pathway.

Other relevant HMO-structures related to brain health are the sialylated HMOs. The most common ones are 3′SL and 6′SL. Sia has shown to be important in neurodevelopment which has been illustrated in several studies and collected in a review by Wang (see review: (Wang, 2009)). As mentioned in previous section, dietary Sia can improve learning and memory in piglets evaluated by the use of 8-arm radial maze (Wang et al., 2007). It has also been evaluated that infants fed infant formula have significantly lower levels of Sia in the frontal cortex compared to breastfed children (Wang, McVeagh, Petocz, & Brand-Miller, 2003). Knowing the structure and prevalence of HMOs, combined with the knowledge that a substrate in human milk is a great source for Sia in the brain, suggests that sialylated HMOs might be a great source of Sia in brain development. The findings of Sia as an important neurodevelopmental modulator points to the need of a Sia source in infants. Recent years, more studies have been performed addressing the sialylated HMOs in the aspect of brain function and brain health.

Oliveros et al., showed that 6′SL improved memory and LTP in rats. The rats receiving Sia and especially the ones receiving 6′SL, did significantly better in the behavioral assessment and showed an enhanced LTP compared to controls (Oliveros et al., 2018). Additionally, Tarr et al., investigated the impact of 3′SL and 6′SL on stressor-induced alterations in the gut microbiota of mice and behavior related to stress. A significant alteration in the colonic microbiota composition of the control mice wase observed, as well as development of anxiety-like behavior. This was not observed in mice fed HMOs, indicating impact of HMOs on behavior related to stress and anxiety (Tarr et al., 2015). Jacobi et al., investigated impact of 3′SL and 6′SL on brain Sia and gut microbiota of neonatal pigs. They observed an increase in ganglioside Sia in the brain of piglets fed the sialylated HMOs and a positive change in the colonic microbiota composition (Jacobi et al., 2015). Recently, Wang et al., showed that sialylated oligosaccharides could increase the amount of several neuroactive biomolecules in piglets (Wang et al., 2019). Even though 3′SL and 6′SL show promising results in relation to brain health and function, Fleming et al., found that sialyllactose (SL) had no effect on recognition memory or diurnal activity in piglets (Fleming, Chichlowski, Berg, Donovan, & Dilger, 2018). Additionally Mudd et al., showed no difference in total and free Sia concentrations in the brain after dietary supplement of SL. However, it was shown that dietary SL in low or moderate concentrations decreased the amount of bound Sia in prefrontal cortex, and moderate concentration of SL increased the ratio of free-to-bound Sia in hippocampus compared to controls (Mudd et al., 2017). It was, however, not indicated which type of SL was used in these studies and it has been demonstrated that the structure complexity is important for brain health. Both studies used 380 mg SL/L (moderate concentration in the study by Mudd et al). These concentrations are lower than the concentration used in other brain-related studies focusing on SL (Jacobi et al., 2015; Wang et al., 2019), indicating that a certain amount of SL is necessary to obtain the beneficial effects. However, in a recent study by Obelitz-Ryom et al., a similar low concentration of SL showed significant results in preterm pigs. The preterm pigs did better on preference test and had increased genes related to Sia metabolism, myelination, and ganglioside biosynthesis in hippocampus. The study showed that SL supplementation did not increaselevels of Sia in hippocampus, and the effect might therefore be related to another microbiota-gut-brain pathway (Obelitz-Ryom et al., 2019). The formulation of the Sia fed through the diet might also be of significant matter, as other Sia-containing compounds have shown a beneficial effect (Wang et al., 2007). The administration of 3′SL and 6′SL did however induce beneficial properties in other studies, and more studies are therefore needed to evaluate if optimization of the kinetics could induce a response. Sialylated HMOs might also affect the microbiota-gut-brain axis through another mechanism than just delivering Sia to the brain, however more studies are needed to fully evaluate the potential of sialylated HMOs as a modulator of the microbiota-gut-brain axis, but the studies performed so far provides a good indication of a positive effect of sialylated HMOs, when given in the right concentrations.

Collectively, these findings show a relation between modulation of the gut microbiota and brain function after digestion of certain HMOs and allow us to suggest that HMOs may impact the brain through the microbiota-gut-brain axis. Several different ways have been suggested and some studies have shown the involvement of the vagus nerve (Vazquez et al., 2016). Studies evaluating the direct effect of HMOs on brain health and function are performed in animals, and of course human trials will need to be performed to fully understand the effect HMOs have on brain health and function. However, the above described experiments indicate that HMOs could be relevant in brain health, even though the specific mode of action is still to be unraveled. HMO-studies investigating brain health or function are summarized in table 2 below.

6. Conclusion

Dietary modulation of the gut microbiota is a promising tool in the management of intra- and extra-intestinally diseases. Inducing a shift in the gut microbiota towards a healthy composition could be a target to improve brain health. A microbial shift could induce beneficial properties via the gut-brain axis in several ways, including the production of bacterial derived biomolecules, or affecting neurologic, immunologic or endocrine pathways directly or indirectly. HMOs are resistant to degradation by host enzymes, and therefore reach the colon undigested, and are available as nutrients for specific gut bacteria. HMOs have long been known to be important for infant health, and are now also known to specifically change the gut microbiota, in particular bifidobacteria in adults. Several animal studies have shown a beneficial impact of HMOs on brain function through the microbiota-gut-brain axis. Even though the specific mode of action of HMOs in brain health still remains to be elucidated, we speculate that HMOs could be a choice for nutritionally managing symptoms related to the CNS.

7. Ethics statements

The research did not include any human subjects or animal experiments.

CRediT authorship contribution statement

Alia H. Al-Khafaji: Conceptualization, Writing - original draft, Investigation, Visualization. **Stine Dam Jepsen:** Conceptualization, Writing - original draft, Investigation, Visualization. **Kristine Rothaus Christensen:** Writing - review & editing. **Louise Kristine Vigsnæs:** Conceptualization, Writing - review & editing, Supervision, Project administration.

Table 2
Literature overview including HMO-studies related to brain health or brain function. Abbreviations used in the table: GF (germ-free); LTP (Long-term potentiation); Sia (sialic acid); SL (sialyllactose); 2′FL (2′-Fucosyllactose); 3FL (3-Fucosyllactose); 3′SL (3′-Sialyllactose); 6′SL (6′-Sialyllactose).

Structure(s)	Concentration + duration	Type of study	Investigation	N	Outcome	Reference
2′FL	1.5uL of 30 mmol solution of 2′FL injected into corpus callosum.	*In vivo*: Wistar rats, 220–240 g, males.	LTP-experiment with intrabrain-electrodes.	N = 40	2′FL enhancedLTP.	(Krug et al., 1994)
2′F, 3FL	60 min bath application of 0.2 mM 2′FL/3FL.	*In vitro*: Brains from 8 weeks, wistar rats, males.	LTP-experiment in brains.	N = 7–10/group.	2′FL increased population spike amplitude and field excitatory postsynaptic potential. 3FL had no effect on LTP.	(Matthies et al., 1996)
3′SL, 6′SL	2/4g 3′SL/6′SL/L equal to 0.6/1.2 g/kg bodyweight or control for 21 days (feeding 3/day).	*In vivo:* Pigs, day-old, full-term crossbred.	Sia analysis in brain tissue and gut microbiota quantification.	N = 54 (4 + 5 pigs pr. treatment).	3′SL and 6′SL altered the gut microbiota and increased ganlioside-bound Sia in the corpus callosum and cerebellum.	(Jacobi et al., 2015)
3′SL, 6′SL	5% of diet was 3′SL or 6′SL for 2 weeks prior to and during stressor.	*In vivo:* C57/BL6 mice, males, 6–8 weeks.	Social disruption stressor, anxiety tests (open field and light/dark preference tests), microbiota sequencing, brain cell proliferation and immature neuronal assessment.	N = 9/group.	3′SL or 6′SL counteracted the stressor induced microbial-change and helped maintain normal behavior on tests of anxiety-like behavior and normal numbers of immature neurons.	(Tarr et al., 2015)
2′FL	Oral administration, through the diet, 350 mg/kg, for 5 weeks (rats) or 12 weeks (mice). In one study, oral gavage, 1 g/kg BW in rats.	*In vivo:* Mice: Male C57BL/6 (2–3.5 months, 25–30 g). Rats: Male Sprague-Dawley (2.5–4 months, 250–300 g).	LTP experiments, IntelliCage study/Skinner box tests, BDNF quatification.	Mice: N = 8–28/group Rats: N = 7–10/group.	2′FL had potentiating effect on hippocampal LTP , improved performance in various learning tests and increased the expression of neuroactive molecules	(Vázquez et al., 2015)
2′FL	350 mg 2′FL/kg of body weight per day.	*In vivo:* Rats: Male Sprague-Dawley (2.5–4 months, 250–300 g).	Electrophysiological studies + vagotomy and Skinner box tests.	N = 10/group.	2′FL improved LTP and operant conditioning. Vagotomy inhibited the effect of 2′FL.	(Vazquez et al., 2016)
SL	From 2 to 32 days of age receiving one of the following diets: Control: 0 mg SL/L, Low: 130 mg SL/L, Moderate: 380 mg SL/L, High: 760 mg SL/L.	*In vivo:* Intact male pigs.	MRI assessement of brain development, Sia measurements in brain and plasma.	N = 38.	In prefrontal cortex a greater proportion of bound Sia was found in control pigs, compared with low and moderate dietary pigs, but were not different between control and high dietary pigs. Ratio of free-to-bound Sia was decreased in moderate dietary pigs in hippocampus.	(Mudd et al., 2017)
SL	From 2 to 32 days of age receiving control diet or SL diet (SL, 190 mg/100 g milk replacer powder ~ 380 mg SL/L).	*In vivo:* Intact male pigs.	Behavioral testing (novel object recognition and activity analysis).	N = 36.	Dietary SL did not alter growth performance, development of recognition memory or gross sleep-related behaviors.	(Fleming et al., 2018)
Sia, 6′SL	Variating doses throughout the experiments: A dose as low as ~ 200 mg/kg/day topping at 2500 mg/kg/day (see Fig. 1d for the exact numbers).	*In vivo:* Pregnant Sprague-Dawley rats + pups (complex setup, see scheme 1 in the paper)	Sia-analysis in brain tissue, LTP-measurements + behavioral and intelligence tests (Novel Object Recognition Test, Y Maze with Blocked Arm Test and IntelliCage®)	N = 47 Complex setup, see scheme 1 in the paper).	Rats receiving Sia, especially as 6′SL, performed better in behavioral assessment and had improved LTP and memory compared to the control group.	(Oliveros et al., 2018)
2′FL	Oral study: 1 g ^{13}C-2′FL/kg body weight or saline as the vehicle via oral gavage. Sacrificed after 0.5, 1, 2, 3, 5, 9, and 15 h (GF mice after 5 + 12 h). Intravenously study in NMRI mice: 200 mg ^{13}C-2′FL/kg body weight every 6 h, for 24.	*In vivo*: Male NMRI mice, 8 weeks of age, 36–47 g or Male C3H/HeN axenic (GF) mice, 6 weeks of age, 29–35 g.	Determination of ^{13}C-2′FL by Elemental Analysis—Isotope Ratio Mass Spectrometry (EA-IRMS)	Oral study: N_GF = 12 N_NMRI = 40. Intravenously_study: N_NMRI = 8.	2′FL did not reach the brain in intact form.	(Kuntz et al., 2019)
SL, 3′SL, 6′SL	433 ± 5 mg/L. 3′SL:6′SL ratio 5:1.	*In vivo:* Preterm pigs, Landrace × Yorkshire × Duroc, born on day 106.	Behavioral tests, MRI and tissue analysis.	N = 46.	SL administration increased cognitive level of preterm pigs to the same as the term pigs and upregulated genes related to sialic acid metabolism	(Obelitz-Ryom et al., 2019)
SL, 3′SL, 6′SL	Sow milk replacer alone or supplemented with SLs 9.5 g/kg (3′SL (7.6 g/kg) and	*In vivo:* Three-day-old male domestic piglets (Sus		N = 15–16/group.	Sialylated milk oligosaccharides altered important brain metabolites and	(Wang et al., 2019)

(*continued on next page*)

Table 2 (*continued*)

Structure(s)	Concentration + duration	Type of study	Investigation	N	Outcome	Reference
	6'SL (1.9 g/kg)) or a combination of SLs and 6'sialyllactosamine 9.5 g/kg (3'SL (7.04 g/kg), 6'SL (1.74 g/kg), 6'sialyllactosamine (0.72 g/kg)) for 35 days.	scrofa – Belgian Landrace, Large White, Landrace and Duroc breed) weighing 1.8–2.2 kg.	Brain spectra were acquired using a 3 T Magnetic Resonance Spectroscopic (MRS) system.		neurotransmitters in piglets, including myoinositol, glutamate and glutamine.	
2'FL	1, 2, 5 and 10% (w/v) in drinking water for six weeks.	*In vivo:* Male C57/BL6 mice, six weeks old.	Diet study: low fat diet vs. high fat diet +/- 2'FL.	N = 6/group.	2'FL modulated the hyperphagic response to high-fat diets, altered the gut microbiota and decreased inflammatory markers infl	(Lee et al., 2020)
2'FL	Pretreatment: 2'FL (30 nmole /15 μg/ animal in 20 μl) intracerebroventricularly. Post-treatment: 2'FL (400 mg/ kg/day) or vehicle, orally fed to the animals once/day from days 2–7 (immunohistochemistry) or from days 2 to14 (bromodeoxyuridine) after middle cerebral artery occlusion	*In vivo:* Adult male Sprague-Dawley rats. *In vitro:* Primary cortical neuronal cells from time pregnant Sprague-Dawley rats (embryos).	Stroke model *in vitro* and *in vivo*. Evaluation of behavioral measurements, infarction, neuroactive biomolecules, neurodegeneration and inflammation	Several different experiments: Group sizes between 6 and 10.	2'FL reduced glutamate-induced neurotoxicity, apoptosis, and Ca++ influx in primary neuronal culture. Pre- or post-treatment with 2'FL reduced brain infarction, neurological and motor deficits and inflammation and increased the expression of BDNF *in vivo*.	(Wu et al., 2020)

Declaration of Competing Interest

Stine Dam Jepsen, Kristine Rothaus Christensen and Louise Kristine Vigsnæs are currently employed at Glycom A/S, a company that produces human milk oligosaccharides. Other than that, there are no known conflicts of interest associated with this publication.

Acknowledgement

The authors acknowledge Claus Heiner Bang-Berthelsen, senior scientist at the National Food Institute, Technical University of Denmark for excellent scientific advice, and Galina Skladtchikova, Arpad Kovacs and Bruce McConnell for their support and the critical review of the manuscript.

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