

Nutritional Neuroscience

An International Journal on Nutrition, Diet and Nervous System

ISSN: 1028-415X (Print) 1476-8305 (Online) Journal homepage: www.tandfonline.com/journals/ynns20

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To cite this article: Md Mahmudul Amin, Tanjina Amin, Adikari Arachchige Dilki Indrachapa Adikari, Xiaoming Zheng, Zhaolin Chen & Bing Wang (2026) Synergistic action of human milk oligosaccharides and lactoferrin enhances neurodevelopment in piglets: evidence from MRS based metabolic profiling, Nutritional Neuroscience, 29:9, 1200-1212, DOI: 10.1080/1028415X.2026.2637610

To link to this article: https://doi.org/10.1080/1028415X.2026.2637610

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Published online: 02 Mar 2026.

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NUTRITIONAL NEUROSCIENCE
2026, VOL. 29, NO. 9, 1200–1212
https://doi.org/10.1080/1028415X.2026.2637610

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Synergistic action of human milk oligosaccharides and lactoferrin enhances neurodevelopment in piglets: evidence from MRS based metabolic profiling

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ABSTRACT

Objective: To investigate the synergistic effects of combined human milk oligosaccharides (HMOs) and lactoferrin (Lf) supplementation on brain metabolites and neurotransmitters in neonatal piglets using proton magnetic resonance spectroscopy (^{1}H-MRS).

Methods: Three-day-old male domestic piglets were randomly assigned to three groups and fed for 35 days with a standard pig milk replacer supplemented as follows: control (methylcellulose, 1.8 g/L; n = 14), combined HMOs (cHMOs, 70% 2′-FL and 30% 3′-SL:6′-SL in a 1:2.5 ratio, 1.8 g/L; n = 16), or cHMOs + Lf (cHMOs, 1.8 g/L + Lf, 0.5 g/L; n = 14). *In vivo* ^{1}H-MRS was performed on postnatal day 38–39 using a 3 T scanner (TE = 30 ms) to assess regional brain metabolite profiles.

Results: The combined cHMOs and Lf group exhibited significantly higher absolute levels of total lipids and macromolecules (tLM), along with increased relative concentrations of glutathione (GSH), total creatine (tCr), total choline (tCho), and tLM ($p < 0.05$). The cHMOs alone group showed a similar upward trend in tCr, tCho, and tLM, but exhibited significantly higher absolute levels of total N-acetylaspartate (tNAA) ($p < 0.05$) compared with the other two groups. Collectively, these findings indicate distinct metabolic profiles among the treatment groups.

Conclusions: The combined cHMOs and Lf supplementation may synergistically support neurodevelopment by enhancing lipid mobilization, energy metabolism, and antioxidant capacity, while cHMOs alone promotes brain development by improving neuronal integrity and synaptic activity in piglets – a translational model for human infants. To our knowledge, these findings have not been previously reported.

KEYWORDS

3′- and 6′-sialyllactose (3′-SL, and 6′-SL); 2′-fucosyllactose (2′-FL); lactoferrin; brain metabolites; MRS; neurodevelopment

Introduction

The first 1,000 days of life – from conception to the first two years – represent the most critical period of neurodevelopment, encompassing rapid phases of synaptogenesis, myelination, and neural circuit refinement [1]. During this window, early-life nutrition plays an impactful and modifiable role in shaping these neurodevelopmental processes. Beyond supporting somatic growth, breast milk provides bioactive molecules that actively modulate neural development and cognitive function [1]. Among these biomolecules, human milk oligosaccharides (HMOs) are the third most abundant components in breast milk after lactose and lipids, comprising over 200 structurally diverse forms, primarily classified as neutral fucosylated, neutral nonfucosylated and sialylated types [2]. HMOs concentrations range from ~20–25 g/L in colostrum to 5–20 g/L in mature milk [3], whereas all currently available infant formulas – predominantly derived from cow's milk – contain oligosaccharides at levels 100–1000 times lower [4]. Although indigestible by infants, HMOs serve as substrates for commensal bacteria, modulating the gut microbiome and promoting the production of neuroactive metabolites such as short-chain fatty acids (SCFAs) [5]. Sialylated HMOs, such as 3′ – and 6′ – sialyllactose (3′-SL and 6′-SL), have been shown in animal models to enhance

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brain development by increasing polysialylation of neural cell adhesion molecules (NCAM), a key regulator of synaptic plasticity and neurogenesis [6] and boosting gangliosides expression as well as changes in hippocampal architecture [4].

To date, most studies on HMO-driven neurodevelopment have focused on fucosylated structures, largely due to limited availability of food-grade sialylated HMOs [3]. Among the sialylated HMOs, 3′-SL has been studied more frequently [7], despite 6′-SL being more abundant and accurately reflects early human milk composition [8]. Given the structural diversity of HMOs, their effects are likely mediated by synergistic interactions. However, few studies have assessed the combined effects of sialylated and neutral HMOs on neurodevelopment, highlighting the need for research on mixed formulations – particularly blends of fucosylated and sialylated type – to more accurately capture the complexity of human milk in preclinical models.

Lactoferrin (Lf) is an iron-binding sialylated glycoprotein and the second most abundant whey protein in human milk [9]. Recently, we demonstrated that Lf promotes neurodevelopment and cognition by upregulating brain-derived neurotrophic factor (BDNF) and polysialic acid (polySia) expression, enhancing hippocampal neurogenesis, and reducing oxidative stress and neuroinflammation – key mechanisms in early brain circuit formation [9,10]. We further showed that low concentrations (~0.5 g/L) support neurodevelopment, while higher doses (~1 g/L) provide enhanced neuroprotection [9]. However, direct evidence of the synergistic effect of HMOs and Lf supplementation on *in vivo* brain biochemistry – particularly in translational models – remains limited.

Proton Magnetic Resonance Spectroscopy (^{1}H-MRS) provides a powerful, non-invasive method for quantifying neurochemical profiles *in vivo*, enabling the detection of key metabolites such as N-acetylaspartate (NAA), glutamate (Glu), choline (Cho), and myo-inositol (mI) – critical markers of neuronal viability, synaptic integrity, and glial function during neurodevelopment [11]. MRS has also been used to detect nutrition-induced changes in metabolites associated with neuronal and glial maturation. Notably, a recent study in neonatal piglets showed that sialylated HMOs altered brain inositol (Ins), glutamine (Gln) and Glu levels, underscoring MRS sensitivity to early nutritional effects [7]. Short echo time (TE, 20–40 milliseconds) – are particularly effective for detecting metabolites with rapid signal decay, enabling broader neurometabolic profiling, and thereby making short-TE ^{1}H-MRS well suited for assessing diet-induced brain changes in translational models of infant development [11].

The piglet is a well-established model for infant brain development due to its gyrencephalic brain, rapid postnatal growth, and comparable neurodevelopmental timelines [6]. Their gastrointestinal physiology closely mirrors that of human infants, supporting studies on the gut-brain axis. Notably, porcine milk contains a more human like oligosaccharides profile – especially sialylated forms – compared to bovine milk [2]. Leveraging these similarities, this study uses ^{1}H-MRS to investigate the impact of HMOs supplementation, alone or in combination with Lf, on piglet brain neurometabolic profiles, providing translational insight into how early dietary interventions may optimize brain development and cognitive outcomes.

Methods

Ethical statement

The research protocol was approved by the Animal Care and Ethics Committee (ACEC) of Charles Sturt University (Project ID: A23566) and Monash University (Project ID: 38776). The study was conducted in compliance with institutional and national guidelines for the care and use of animals in research.

Animal model and housing

Male domestic piglets (*Sus scrofa* – Large White x Duroc x Belgian Landrace breed) were sourced from a commercial breeding facility (Pig Improvement Company, Grong Grong, NSW, Australia) at postnatal day (PND) 3 (n = 48; body weight 1.5–2.6 kg). Piglets were stratified by body weight and litter origin, then randomly assigned to one of three experimental groups (Blue, Yellow, and Purple). The piglets were housed in pairs within temperature-controlled pens (2.2 m × 2.5 m), each equipped with environmental enrichment, including a plastic toy, a towel-covered rubber tire nest and a heat lamp. Ambient temperature was maintained at 27°C initially and gradually reduced to 23–25°C by PND 21. A 12 h

light/12 h dark cycle (08:00–20:00) maintained throughout the study. During the first two weeks of the study, four piglets were lost (two from the control group and two from the cHMOs + Lf group; body weight range: 1.7–1.8 kg). One piglet died due to intussusception, one was euthanized because of multi-organ dysfunction, and two died from pneumonia and septicaemia, resulting in a final cohort of 44 animals. All research personnel were blinded to treatment-group allocation. The experimental procedure is illustrated in Figure 1.

Animal feeding

Piglets were fed a standard sow milk replacer (soy: whey: casein ratio of 50:38:12) from PND 3–38/39, which is equivalent to 10–12-month-old human infants [9]. Three experimental diets were prepared by blending 3′-SL and 6′-SL (Zhongke Hesheng, China), 2′-FL (Yixi Biotech, China), or Lf (Saputo Dairy Australia) into pig milk replacers (Wombaroo Food Products, Australia) at specified concentrations for each group. The final milk concentrations varied according to groups: the cHMOs group received 1.8 g/L cHMOs (70% 2′-FL and 30% sialylated HMOs with a 3′-SL to 6′-SL ratio of 1:2.5; n = 16); the combined cHMOs + Lf group received 1.8 g/L cHMOs and 0.5 g/L Lf (n = 14), and the control group received 1.8 g/L methyl cellulose (n = 14). The nutritional composition of all diets is detailed in Table 1. Formulations were designed to ensure comparable nutrient intake across groups, with HMOs and Lf – as the only differing components, as confirmed by validated analytical methods [2,12] (Table 2).

Piglet feeding and husbandry followed established protocols [4,9]. To maintain normal growth rates, the piglets were fed milk replacers at 285 mL/kg/day during the first two weeks, followed by 230 mL/kg/day in the subsequent weeks. Feeding occurred four times daily (08:00, 13:00, 18:00, and 22:30), with an additional 50 mL provided at the last feeding. Body weight was recorded daily prior to morning feeding using a digital scale (MS2200, A-Bame technology, China), and milk intake and health status were monitored throughout the study.

MRS data acquisitions

All piglet brain neuroimaging was performed on PND 38 or 39, using a Siemens Skyra 3 T MR scanner (Siemens Skyra, Erlangen) equipped with a 15-channel radiofrequency (RF) coil. Prior to ^{1}H-MRS, piglets were fasted for at least 4 h. Anaesthesia was induced with 5% isoflurane in medical-grade oxygen for 3–5 min and maintained with 2–3.5% during imaging to minimize motion artifacts [7]. Vital signs including heart rate, respiratory rate, and oxygen saturation were continuously monitored and recorded every 5 min using a tail-mounted sensor connected to the monitoring system (Nonin 7500FO Pulse Oximeter, Nonin Medical Inc, MN, USA). The total scan duration for each piglet was approximately 45 min.

MRS data acquisitions were performed using our previously published protocol [7]. Briefly, ^{1}H-MRS was performed using a single-voxel spin echo point resolved spectroscopy (PRESS) sequence (TR = 2000 ms; TE = 30 ms) with the voxel positioned in the frontal lobe grey and white matter (15 mm left-to-right, 14 mm

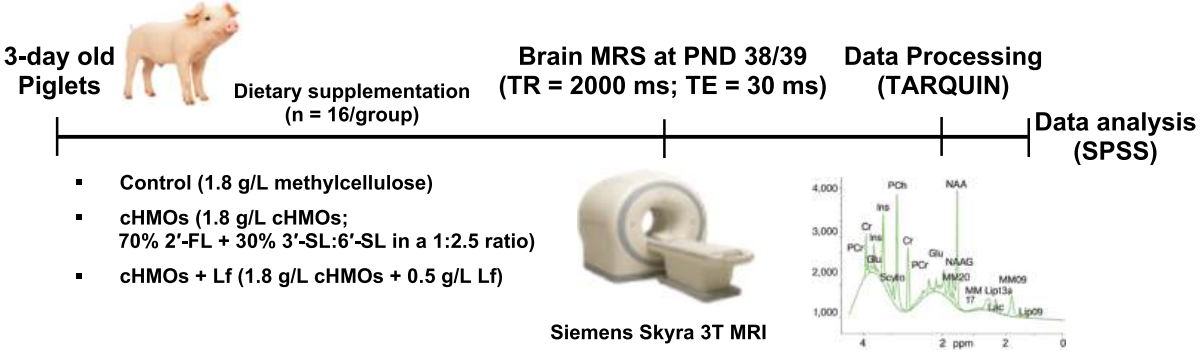


Figure 1. Schematic depiction of the experimental design. Three-day-old male piglets were fed a sow milk replacer supplemented with cHMOs, cHMOs + Lf, or control for 35–36 days. On postnatal day 38–39, piglets underwent MRS, and data were processed by TARQUIN software.

Table 1. Nutritional composition (% unless otherwise noted) of commercial (control) and modified pig milk replacers.

Component	Control	cHMOs	cHMOs + Lf
Min. Crude Protein (%db)	31%	31%	30.722% Lf = 0.278%
Min. Crude Fat (%db)	40%	40%	40%
Carbohydrate (%db)	18% Methyl cellulose = 1%	17% 2′-FL = 0.70% 3′-SL = 0.086% 6′-SL = 0.214%	17% 2′-FL = 0.70% 3′-SL = 0.086% 6′-SL = 0.214%
Metabolisable Energy (ME)	4200 kJ/Litre	4200 kJ/Litre	4200 kJ/Litre
Max. Fibre	0%	0%	0%
Min. Calcium (Ca)	1.20%	1.20%	1.20%
Max. Calcium (Ca)	1.60%	1.60%	1.60%
Min. Phosphorus (*P*)	0.90%	0.90%	0.90%
Max. Phosphorus (*P*)	1.30%	1.30%	1.30%
Min. Salt (NaCl)	0.70%	0.70%	0.70%
Max. Salt (NaCl)	1.10%	1.10%	1.10%
Min. Lysine	2.30%	2.30%	2.30%
Min. Vitamin A	9 mg/kg	9 mg/kg	9 mg/kg
Min. Vitamin D3	90 µg/kg	90 µg/kg	90 µg/kg
Min. Vitamin B2	9 mg/kg	9 mg/kg	9 mg/kg
Min. Magnesium (Mg)	500 mg/kg	500 mg/kg	500 mg/kg
Min. Ferrous Iron (Fe^{2+})	90 mg/kg	90 mg/kg	90 mg/kg
Min. Zinc (Zn)	15 mg/kg	15 mg/kg	15 mg/kg
Min. Manganese (Mn)	10 mg/kg	10 mg/kg	10 mg/kg
Min. Selenium (Se)	120 µg/kg	120 µg/kg	120 µg/kg

Note: db = dry matter basis; Ingredients – Whole milk solids, whey protein isolates, milk fat, vegetable oils, vitamins and minerals.

superior-to-inferior, 20 mm anterior-to-posterior) (Figure 2). All imaging procedures were conducted by a professional imaging scientist. Following MRS, the piglets were euthanized via intracardiac injection of lethabarb (1–2 mL, 80 mg/kg body weight).

MRS data analysis

^{1}H-MRS data were processed using TARQUIN software (version 4.3.10), which enables automated quantification of metabolites from both long and short echo-times [13]. Analysis was performed in the time domain using the 'advanced fit' option with a non-negative least-squares fitting algorithm. Eddy current correction was applied to the water-suppressed spectra and metabolite concentrations were calculated in institutional units without further T2 relaxation correction [7] (Figure 3).

Statistical analysis

Group comparisons of individual brain metabolite concentrations were conducted using a univariate general linear model (GLM), adjusting for covariates including initial body weight, cerebrum weight, faecal score, and milk intake. Metabolite concentrations were expressed both as absolute values and as ratios to tNAA. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed in IBM SPSS Statistics (Version 29.0.0.0; SPSS Inc., Chicago, IL, USA).

Results

The absolute concentration of brain metabolites

The absolute concentrations of 33 brain metabolites including alanine (Ala), aspartate (Asp), creatine (Cr), γ-aminobutyric acid (GABA), glycerophosphorylcholine (GPC), d-Glucose (Glc), Gln, glutathione (GSH),

Table 2. Analyzed concentrations of oligosaccharides (µg/mL).

Title	Control	cHMOs	cHMOs + Lf
3′-Sialyllactose	57.28	151.02	222.42
6′-Sialyllactose	93.91	188.61	279.21
2′-Fucosyllactose	18.64	1083.02	1157.30

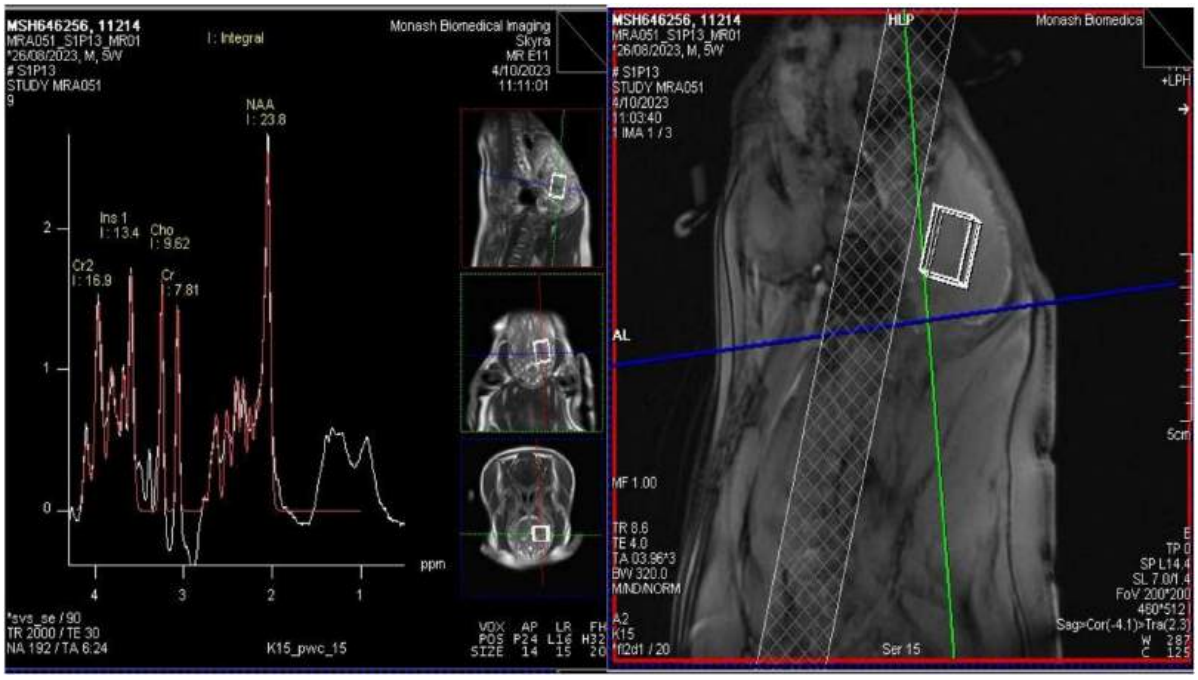


Figure 2. Position of the voxel of interest in the scanner console (frontal lobe grey and white matter; 15 mm left-to-right, 14 mm superior-to-inferior, 20 mm anterior-to-posterior) for brain ^{1}H-MRS in the piglet at postnatal day 38–39.

Glu, Ins, lactate (Lac), lipid (Lip) (09, 13a, 13b, 20), macromolecules (MM) (09, 12, 14, 17, 20), NAA, N-acetylaspartylglutamate (NAAG), phosphorylcholine (PCh), phosphocreatine (PCr), scyllo-inositol (SI), taurine (Tau), total NAA (tNAA), total choline (tCho), total creatine (tCr), glutamate + glutamine (Glx), total lipid and macromolecules (tLM) (09, 13, 20) were automatically quantified using TARQUIN software across all three piglet groups. The mean absolute concentrations of 33 metabolites in 3 different groups of 38/39 d-old piglets are shown in Figure 4.

Among the 33 brain metabolites analyzed, the absolute tLM concentrations were highest in the combined cHMOs + Lf group (173.25 ± 29.78 mM), followed by the cHMOs group (146.88 ± 9.93 mM) and the control group (132.52 ± 10.01 mM). The overall difference among the groups was statistically significant ($P < 0.05$), with post hoc analysis revealing a significant difference between the cHMOs + Lf and the control

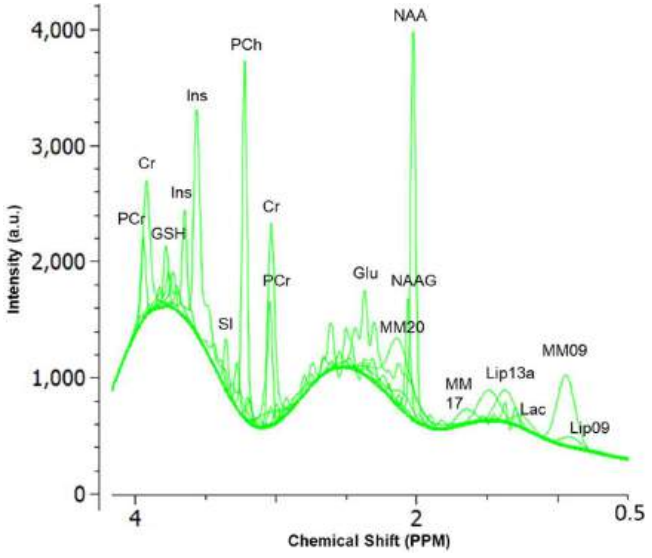


Figure 3. *In vivo* ^{1}H-MR Spectra acquired at TE 30 ms in TARQUIN software.

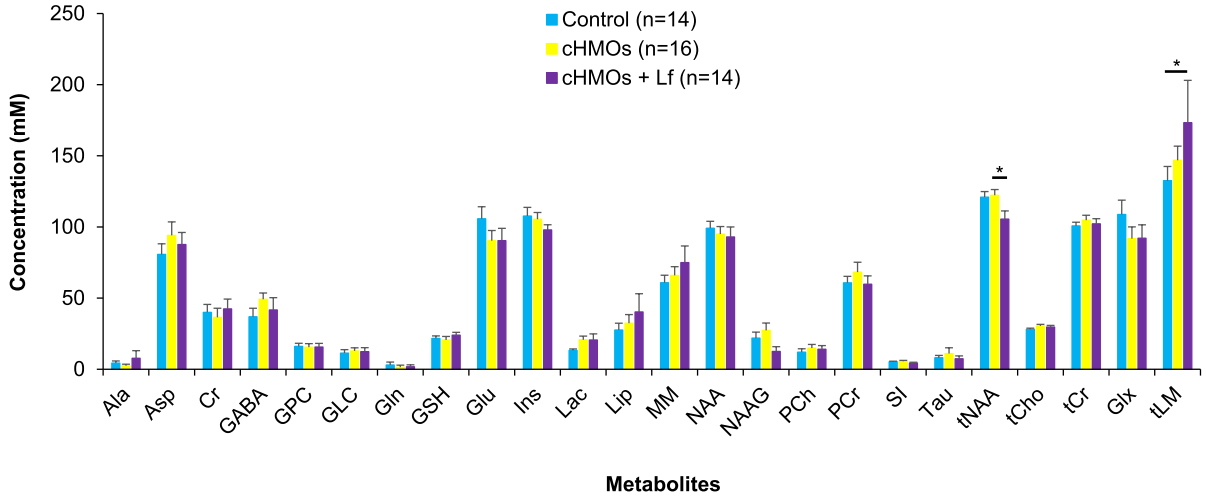


Figure 4. The mean (±SE) absolute concentration of 24 metabolites in 3 different groups at TE 30 ms. Alanine (Ala), aspartate (Asp), creatine (Cr), γ-aminobutyric acid (GABA), glycerophosphorylcholine (GPC), d-Glucose (Glc), glutamine (Gln), glutathione (GSH), glutamate (Glu), inositol (Ins), lactate (Lac), lipid (Lip) (09, 13a, 13b, 20), macromolecules (MM) (09, 12, 14, 17, 20), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG),, phosphorylcholine (PCh), phosphocreatine (PCr), scyllo-inositol (SI), taurine (Tau), total NAA (tNAA), total choline (tCho), total creatine (tCr), glutamate + glutamine (Glx), total lipid and macromolecules (tLM) (09, 13, 20). *$P < 0.05$, General linear model (univariate).

group ($P < 0.05$, Figure 4). The increased tLM concentration in the combined cHMOs + Lf group imply enhanced lipid metabolism, a process often associated with brain development and maturation, as well as enhanced myelination, neuronal membrane synthesis, and energy metabolism [9,14]. Furthermore, the cHMOs + Lf group demonstrated elevated levels of Ala (78–211%), Cr (6–16%), GSH (10–16%), Lip (24–46%), and MM (14–23%), however, the overall differences among the groups did not reach statistical significance (Figure 4).

In contrast, tNAA concentrations were highest in the cHMOs alone group (122.32 ± 3.98 mM), followed by the control (120.98 ± 3.90 mM) and then the combined cHMOs + Lf groups (105.54 ± 5.75 mM). The overall group effect was significant ($P < 0.05$), with post hoc analysis confirming a significant difference between the cHMOs and cHMOs + Lf groups ($P < 0.05$). The elevated tNAA concentration in the cHMOs group may indicate enhanced neuronal integrity or metabolic function, consistent with the established role of NAA in brain metabolism [15].

In addition, the cHMOs alone group exhibited the highest concentrations of several metabolites related to neuronal function and energy metabolism, including Asp, GABA, Glc, Lac, NAAG, PCh, PCr, SI, Tau, tCho, and tCr – although these differences did not reach statistical significance. Although not statistically significant, the 18–33% higher GABA levels in the cHMOs group compared to the cHMOs + Lf and control groups suggest enhanced inhibitory neurotransmission, potential neurodevelopmental benefits, and neural function improvement [16], highlighting a distinct effect of cHMOs supplementation alone on neurotransmitter metabolism.

The relative concentration of brain metabolites (as ratios to tNAA)

We normalized metabolite concentrations to tNAA, which serves as a robust marker of neuronal integrity and density and is relatively stable across physiological and nutritional conditions in the developing brain, making it a more appropriate internal reference in this context. Given the established role of tNAA as a marker of neuronal metabolism and integrity, tNAA-normalized metabolite ratios provide the most biologically relevant framework for interpreting diet-induced neurodevelopmental effects [17]. This approach is supported by extensive MRS literature showing that NAA or tNAA is frequently used as a normalization reference, particularly in studies involving development, metabolism, and nutritional intervention-based designs [17–19]. Moreover, tNAA normalization has also been applied in paediatric and neonatal MRS studies where it has been shown to accurately predicts motor, cognitive and language outcomes in neonates

[20]. In contrast, evidence from disease-focused MRS studies, including HIV-associated dementia, demonstrates that total creatine – often assumed to be stable and used as a reference metabolite – can vary significantly across brain regions and with disease severity [21]. Given evidence that creatine may vary with pathology and brain region, tNAA – an established marker of neuronal integrity – was selected as a more stable and biologically relevant normalization reference for interpreting neurodevelopmental effects. This normalization approach is essential for minimizing inter-individual variability caused by differences in brain size, tissue density, and water content, thereby improving the comparability of metabolite profiles across groups.

Among the 33 brain metabolites analyzed, the mean relative concentrations (ratios to tNAA) of tCho, tCr, and tLM were significantly higher in the cHMOs + Lf group (0.29 ± 0.01, 0.99 ± 0.04, and 2.39 ± 0.22, respectively), followed by the cHMOs group (0.25 ± 0.01, 0.87 ± 0.03, and 1.72 ± 0.12, respectively) and then the control group (0.23 ± 0.01, 0.84 ± 0.03, and 1.53 ± 0.11, respectively); the overall differences among the groups were statistically significant ($P < 0.05$, Figure 5). The GSH concentration was highest in the cHMOs + Lf group (0.23 ± 0.02), followed by the control group (0.18 ± 0.01) and the cHMOs group (0.17 ± 0.02). The overall difference among the groups was statistically significant and post hoc analysis identified a significant difference between the cHMOs + Lf and the cHMOs group ($P < 0.05$). These differences became more pronounced after adjusting for initial body weight, cerebrum weight, milk intake, and faecal score, underscoring a robust association between Lf supplementation and increased metabolite levels ($P < 0.05$). Functionally, GSH serves as a key antioxidant supporting neuronal protection and redox balance [22]; tCho reflects membrane turnover and cholinergic neurotransmission [23]; tCr functions as an energy buffer sustaining ATP homeostasis [24]; and tLM represents lipid and macromolecule content associated with myelination and brain maturation [14]. Collectively, the elevation of these metabolites in both the cHMOs + Lf and cHMOs groups suggests that supplementation with cHMOs, either alone or in combination with Lf, may enhance neurodevelopment, neuroprotection, and energy metabolism; further behavioral and tissue-based mechanistic studies would help extend these findings.

The cHMOs + Lf group displayed higher concentrations of several major brain metabolites, including Asp (14–30%), Ins (6–9%), PCr (3–15%), GPC (12–15%), and NAA (5–11%) compared to the cHMOs and control groups, however, these differences did not reach statistical significance ($P > 0.05$). These metabolites play key roles in neurodevelopmental processes, including cellular signaling, postnatal myelination

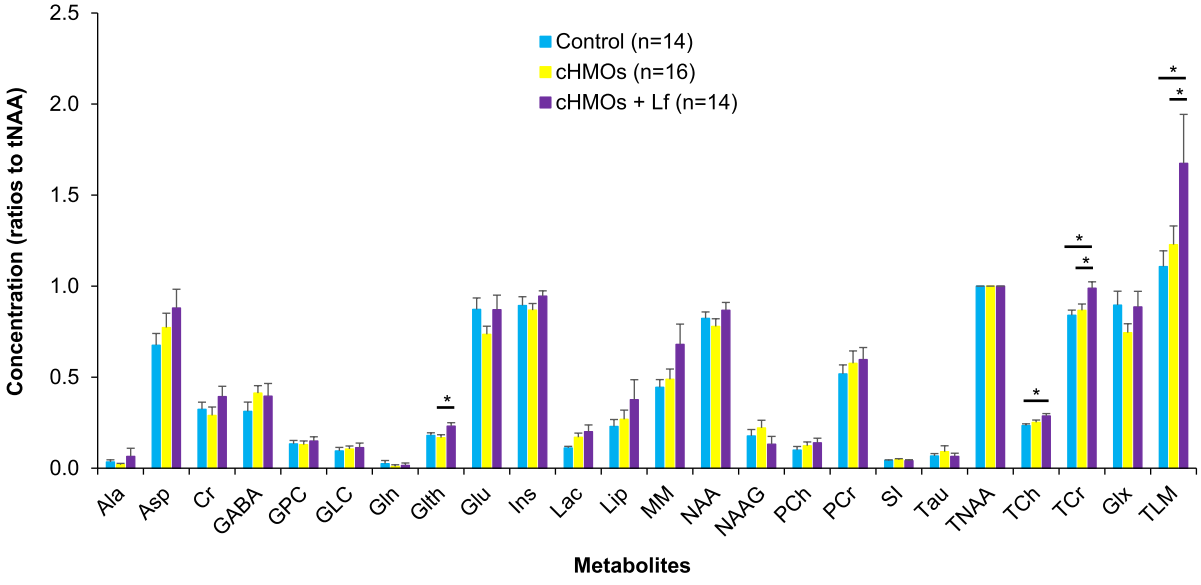


Figure 5. The mean (±SE) relative concentrations (as ratios to tNAA) of each brain metabolites at TE 30 ms in 3 different groups including alanine (Ala), aspartate (Asp), creatine (Cr), γ-aminobutyric acid (GABA), glycerophosphorylcholine (GPC), d-Glucose (Glc), glutamine (Gln), glutathione (GSH), glutamate (Glu), inositol (Ins), lactate (Lac), lipid (Lip) (09, 13a, 13b, 20), macromolecules (MM) (09, 12, 14, 17, 20), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG),, phosphorylcholine (PCh), phosphocreatine (PCr), scyllo-inositol (SI), taurine (Tau), total NAA (tNAA), total choline (tCho), total creatine (tCr), glutamate + glutamine (Glx), total lipid and macromolecules (tLM) (09, 13, 20). *$P < 0.05$, General linear model (univariate).

and neuroprotection, suggesting a potential neurodevelopmental advantage linked to the combined supplementation of HMOs and Lf [7].

Conversely, the cHMOs group exhibited notably higher levels of SI with concentrations 11–15% higher than those in both the control and cHMOs + Lf groups. SI is involved in enhanced neurogenesis and may provide neuroprotective effects by supporting cellular function and preventing protein aggregation [25], suggesting that cHMOs supplementation alone may specifically mitigate neuronal toxicity and preserve synaptic function – key processes in brain development.

Discussion

Our study provides first scientific evidence on the synergistic effects of combined HMOs and Lf on brain metabolite and neurotransmitter profiles in neonatal piglets, revealing distinct metabolic response between HMOs alone and HMOs combined with Lf that may support neurodevelopment. The cHMOs + Lf group exhibited the highest tLM levels in both absolute and relative concentrations compared to the cHMOs group, while the lowest were observed in the control group ($P < 0.05$). As tLM represents lipid and macromolecule content, these elevations suggest enhanced myelination, neuronal membrane synthesis and lipid metabolism – processes critical for ongoing brain maturation rather than direct evidence of de novo myelination [14]. Furthermore, the enhanced lipid mobilization may be attributable to Lf's modulatory effects on lipid synthesis and turnover [26]. While Lf is known to reduce lipid accumulation in peripheral tissues, its role in the brain may involve modulating lipid turnover and redistribution rather than simple storage [10]. The observed increase in tLM likely reflects adaptive lipid remodeling processes essential for neurodevelopment, including membrane biosynthesis, myelination, and energy homeostasis, rather than pathological lipid deposition. Accordingly, this increase should be interpreted as indicative of altered lipid and macromolecular metabolism rather than direct quantification of individual lipid species. To our knowledge, the findings have not been previously reported.

We also demonstrated that the relative concentrations of tCr and tCho followed a pattern similar to tLM: the combined Lf + HMOs group showed the highest levels, the HMOs group had intermediate levels, and the control group exhibited the lowest levels ($P < 0.05$). Creatine plays a vital role in brain energy metabolism primarily through the phosphocreatine (PCr) system, which supports ATP regeneration. It also enhances cognitive function by facilitating neurotransmitter synthesis, modulating synaptic plasticity, and protecting neurons from oxidative stress [24]. Choline is essential for acetylcholine and betaine synthesis, contributes to osmotic regulation and serves as a precursor for phospholipids, sphingolipids and lecithin, which are vital for membrane integrity and myelination [7]. Elevated tCho level reflects increased membrane phospholipid turnover, consistent with active structural growth, myelination, and enhanced membrane integrity in the developing brain [23]. In parallel, higher tCr levels indicate improved energy storage and transfer, supporting high metabolic demands during brain growth or stress adaptation [24]. Collectively, these findings suggest that Lf in combination with cHMOs may promote cognitive development, neuroprotection, neurostructural maturation and overall brain health.

Unexpectedly, the cHMOs + Lf group showed significantly higher GSH levels compared with both the cHMOs and control groups ($P < 0.05$), whereas the cHMOs group exhibited the lowest concentrations. The elevation suggests enhanced antioxidant capacity and improved cellular protection through strengthened redox homeostasis [22]. Beyond its role in redox balance, GSH also contributes to detoxification of xenobiotics, amino acid transport, and regulation of protein function. It protects against cysteine and glutamate toxicity and modulate glutamatergic signaling which is critical for synaptic transmission and neural plasticity. Emerging evidence indicates that GSH may function as a neurotransmitter, acting on specific receptors and potentially influencing learning and memory processes [22]. The observed pattern indicates that Lf may synergistically with HMOs enhance neuroprotective effects by modulating oxidative balance during rapid brain growth. Collectively, these findings suggest that combined cHMOs and Lf supplementation may promote brain maturation, energy metabolism, antioxidant defence, and structural development during a critical neurodevelopmental window. These results reinforce the concept that early-life nutrition can shape neurochemical pathways, potentially leading to enduring functional benefits. To our knowledge, the findings have not been previously reported.

Additionally, the cHMOs + Lf group exhibited 6–9% higher levels of Ins (or myo-inositol) compared to the cHMOs and control groups. Myo-inositol is crucial in the CNS for cellular signaling processes and

serves as a marker of neuroglial cells, particularly in astrocytes. It also plays a key role in fetal brain development and osmotic regulation. Beyond supporting membrane structure, vesicular trafficking, cell survival, and sexual maturation, myo-inositol also serves as a precursor for inositol lipids and phosphates that regulate proliferation, differentiation, and apoptosis [7]. Similar increases in brain Ins have previously been reported in piglets supplemented with combined SL and 6′-SLN, suggesting that HMOs can influence inositol-related metabolic pathways during neurodevelopment [7]. Notably, sialylated HMO supplementation has been reported to upregulate expression of glial fibrillary acidic protein (GFAP), a canonical astrocyte marker, indicating modulation of astrocytic state [27]. Importantly, increased GFAP expression correlates with elevated brain Ins levels, consistent with myo-inositol being enriched within astrocytes and reflective of astrocytic metabolic and osmotic activity [27]. Moreover, Lf has established roles in early neurodevelopmental programs, including activation of BDNF signaling, enhanced polysialylation, and in preclinical models it modulates brain metabolic outcomes and glial responses [9]. Together, these findings support the interpretation that the higher myo-inositol observed in the cHMOs + Lf group may reflect synergistic effects of HMOs and Lf on astrocyte-associated metabolic processes.

In the cHMOs + Lf group, the lack of an increase in absolute NAA concentration occurred alongside elevations in other metabolites related to energy metabolism (Cr), membrane turnover (tCho), and redox regulation (GSH). This pattern suggests that neuronal metabolic demands may be supported through alternative or complementary metabolic pathways rather than through increased NAA levels alone. Moreover, the relative concentration of NAA (expressed as a ratio to tNAA) was highest in the cHMOs + Lf group, indicating that NAA remains proportionally abundant within the broader metabolic context. Taken together, these findings suggest a redistribution of metabolic resources rather than impaired neuronal function.

The cHMOs-only group exhibited the highest concentration of tNAA, a marker of neuronal density and viability compared to both the cHMOs + Lf and control groups. Although NAA has traditionally been linked to myelination and carbon transfer via aspartoacylase, it is increasingly recognized for its role in cellular signaling, particularly in regulating histone acetylation [15]. Additionally, elevated levels of NAAG – a modulator of glutamate release and promoter of synaptic plasticity and neuroprotection – were observed in the cHMOs group [7]. Collectively, these findings imply that cHMOs alone may specifically support neuronal integrity and viability, enhance neuronal metabolism, and promote synaptic function, indicating a distinct neurodevelopment and neuroprotective effect independent of Lf supplementation. To our knowledge, the findings have not been previously reported.

Furthermore, the cHMOs group exhibited 18–33% higher GABA levels, a key inhibitory neurotransmitter that maintains synaptic balance and supports cognitive processes [16]. Additionally, higher levels of SI, a metabolite associated with reduced amyloid aggregation, synaptic integrity preservation, and enhanced neurogenesis were observed in the cHMOs group [25]. Collectively, these findings suggest that cHMOs alone may enhance neurodevelopmental stability through modulating multiple metabolic pathways. The proposed mechanisms by which cHMOs and cHMOs + Lf supplementation influence brain metabolism and neurotransmission are shown in Figure 6.

The dosages of HMOs and Lf used in this study were carefully selected based on previous research demonstrating their neurodevelopmental and metabolic effects [3,9]. The dose of sialylated HMOs (3 g/kg or 540 mg/L) was derived from the average concentration of 3′-SL and 6′-SL during lactation [3], with a 3′-SL:6′-SL ratio of 1:2.5 consistent with levels in mature human milk [3,8]. The 2′-FL dose (7 g/kg) was based on the typical distribution of neutral (50–70%) and sialylated (10–30%) HMOs in human milk [2]. The Lf dose (2.78 g/kg) was carefully chosen to mirror the natural levels found in mature human milk and sow's transitional milk, ensuring physiological relevance across both species [12]. To standardize fiber content and minimize confounding effects on the gut microbiota, methyl cellulose was administered at a dose equivalent to the total HMOs (10 g/kg) as the control group [28].

Previous studies have reported mixed results on the effects of HMO supplementation on neurotransmitter and brain metabolite profiles in piglets using ^{1}H-MRS. Mudd et al. [29] investigated the effects of a bovine-derived modified whey protein enriched SL at varying concentrations (0, 130, 380, and 760 mg/L) over 30 days and found no significant alterations in brain metabolite profiles [29]. Notably, Mudd's formulation included DHA (174 mg/L), ARA (348 mg/L), GOS (2 g/L), and PDX (2 g/L) in both the control and treatment diet that may have confounded the isolated effects of SL on neurometabolite levels. Similarly,

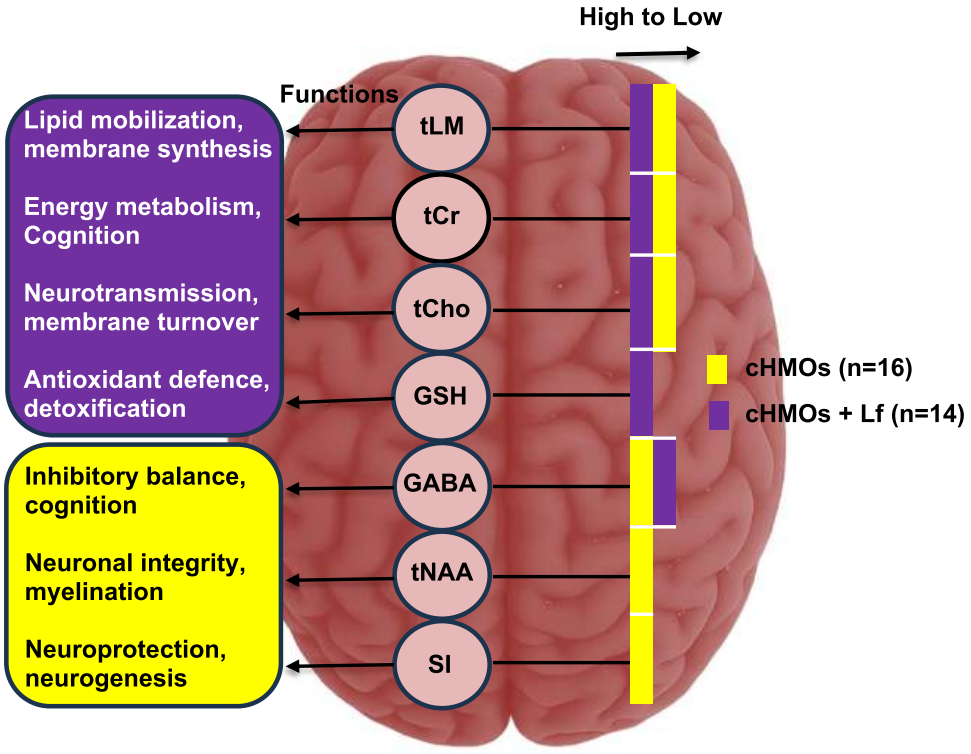


Figure 6. Proposed mechanisms by which cHMOs and cHMOs + Lf supplementation influence brain metabolism and neurotransmission during neurodevelopment. cHMOs alone elevate tNAA, supporting neuronal integrity and synaptic plasticity. cHMOs + Lf increase tLM, tCr, and GSH, enhancing lipid metabolism, energy buffering, and antioxidant defence. Both interventions raise tCho, GABA, and SI, promoting cholinergic neurotransmission, neurotransmitter balance, and neuroprotection.

administration of 2′-FL in combination with oligofructose (OF) or bovine milk oligosaccharides (BMOS) did not result in significant alterations in overall metabolic state [30,31]. Both Fleming and Mudd used a spin-echo chemical shift sequence targeting the dorsal hippocampus (voxel size: $20 \times 23 \times 13$ mm^3; TR/TE: 1,800/68 ms for water-suppressed scans). In contrast, Wang et al. [7] utilized a PRESS sequence centered over the frontal cortex and white matter (voxel size: $15 \times 14 \times 20$ mm^3; TR/TE: 2,000/270 ms) and reported significantly increased levels of Glu, mIns, and Glx following pure SL and 6′-sialyllactosamine (6′-SLN) supplementation. Moreover, the mean absolute concentrations of tNAA, PCr, SI, and tCho were 2–15% higher in the treatment groups compared to the control group [7]. Variability across studies also reflect differences in dosage, timing of assessment, and analytical criteria such as the use of Cramer – Rao lower bounds [29–31]. Addressing these factors is essential to draw consistent conclusions regarding HMO effects on brain metabolism.

While this study employed a double-blind randomized controlled design and carefully minimized confounding variables, several limitations should be considered. The relatively small and unequal sample sizes across the groups (n = 14∼16) may have reduced statistical power and limited the generalizability. Notably, the loss of two low-body-weight piglets in the control group resulted in a final cohort composed solely of high-body-weight animals, limiting direct comparisons with average-weight treatment groups. Early postnatal diarrhea, a common and biologically relevant challenge in neonatal piglets – that closely mirrors vulnerability in human infants – significantly influenced several brain metabolite concentrations ($P < 0.05$), potentially confounding treatment effects. The study focused on *in vivo* neurochemical and metabolic outcomes following dietary intervention using non-invasive MRS and therefore did not include behavioral assessments or detailed analyses of cerebrospinal fluid, blood biomarkers, or region-specific brain lipid composition. Likewise, specific lipid species such as gangliosides, phospholipids, or milk fat globule membrane components were not directly quantified, even though these molecules are known to contribute to neurodevelopmental processes. Accordingly, the observed metabolic changes should be interpreted within

the context of early postnatal brain maturation rather than fetal development. Nevertheless, the metabolic profiles obtained reflect biologically meaningful processes of early postnatal brain maturation, supporting the translational relevance of this model. Future studies with larger, more balanced cohorts that integrate behavioral outcomes, molecular biomarkers, and region-specific lipidomic analyses will be required to clarify the neurodevelopmental effects of combined cHMOs and Lf supplementation.

Conclusion

This study provides the first evidence that early-life supplementation with cHMOs alone or in combination with Lf modulates brain metabolite and neurotransmitter profiles in neonatal piglets, thereby promoting neurodevelopment. The combined cHMOs and Lf significantly enhanced key brain metabolites, including tLM, GSH, tCr, and tCho, suggesting a synergistic effect on neurotransmitter synthesis, energy metabolism, and lipid mobilization supporting myelination, neuronal protection, and structural brain development. cHMOs alone primarily elevated metabolites associated with neuronal integrity, neurotransmitter balance and neuroprotection, including tNAA, GABA, and SI, indicating distinct neurodevelopmental benefits independent of Lf. Our findings provide a foundation for understanding the distinct mechanisms by which HMOs alone versus HMOs combined with Lf improve neurodevelopment and cognition in developing animals; however, their long-term cognitive and neurodevelopmental effects warrant further investigation.

Acknowledgments

We greatly acknowledge Dr. Michael de Veer, Mr. Richard McIntyre, and Dr. Hongxin Wang from Monash Biomedical Imaging, Monash University, for their assistance with MRS data acquisition. We also thank Dr. Trish Holyoake and Dr. Tina Bryant, a registered specialist veterinary anaesthetist, for their assistance with pig anaesthesia. In addition, we gratefully acknowledge Dr. Wentian Li, Ms. Lucy Walsh, and Dr. Zahra Naqvi for their help with feeding the piglets. We also acknowledge the Charles Sturt University (CSU) PhD scholarship awarded to Md Mahmudul Amin.

Author contributions

MMA: Collected MRS data, performed data analysis and interpretation, and drafted the initial manuscript, TA: Animal care, feeding, and euthanasia, AADIA: Animal care, feeding, and euthanasia, XZ: Supervision, data analysis, validation, review & editing, ZC: Supervision, validation, review, BW: Conceptualized and designed the study, analyses and interpretation of the results, supervision, review, editing and final approval of the manuscript for publication.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research is funded by Junlebao Dairy Group Co., Ltd. (China). The study design, data collection, analysis, interpretation of results, and manuscript preparation were made independently of Junlebao.

Generative AI statement

The authors declare that no AI tool was used to draft this manuscript.

Data availability statement

The data are available from the corresponding author upon reasonable request.

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