*British Journal of Nutrition* (2022), 128, 1906–1916 doi:10.1017/S0007114521005146


# Effect of supplementation with select human milk oligosaccharides on artificially reared newborn rats

Weilan Wang[1], Chunlong Mu[2], Nicole A. Cho[1], Erin W. Noye Tuplin[1], Dana E. Lowry[1], Faye Chleilat[1], Kate M. Sales[1], Kara Sampsell[1], Jane Shearer[1,2] and Raylene A. Reimer[1,2]*

[1]*Faculty of Kinesiology, University of Calgary, Calgary, AB T2N 1N4, Canada*
[2]*Department of Biochemistry & Molecular Biology, Cumming School of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada*

*(Submitted 16 September 2021 – Final revision received 2 December 2021 – Accepted 21 December 2021 – First published online 29 December 2021)*

## Abstract

Early life nutrition fundamentally influences neonatal development and health. Human milk oligosaccharides (HMO) are key components of breast milk but not standard infant formula that support the establishment of the newborn gut microbiota. Using an artificial rearing system, our objective was to test the effect of two HMO on the whole body and organ growth, adiposity, glucose tolerance and faecal microbiota in young rat pups. From postnatal days 4 to 21, Sprague–Dawley rats were randomised to receive one of: (1) CTR (rat milk substitute); (2) 2′FL (CTR + 1·2 g/l 2′-fucosyllactose); (3) 3′SL (CTR + 1·2 g/l 3′-sialyllactose) and (4) 2′FL + 3′SL (CTR + 0·6 g/l 2′-FL + 0·6 g/l 3′-SL). Body weight (BW), bowel movements and food intake were monitored daily, faecal samples collected each week and oral glucose tolerance, body composition and organ weight measured at weaning. No significant differences were observed between groups in growth performance, body composition, organ weight and abundance of dominant faecal microbes. A decreased relative abundance of genus *Proteus* in week 1 faecal samples and *Terrisporobacter* in week 3 faecal samples ($P < 0{\cdot}05$) was suggestive of a potential pathogen inhibitory effect of 3′SL. Longitudinal changes in the faecal microbiota of artificially reared suckling rats were primarily governed by age ($P = 0{\cdot}001$) and not affected by the presence of 2′-FL and/or 3′-SL in rat milk substitutes ($P = 0{\cdot}479$). Considering the known protective effects of HMO, further investigation of supplementation with these and other HMO in models of premature birth, extremely low BW or malnutrition may show more pronounced outcomes.

**Key words:** **Artificial rearing: Newborn rats: Human milk oligosaccharide: Gut microbiota**

The establishment of the intestinal microbiota during and after birth is critical for the maturation and development of important body systems, including the digestive, immune and central nervous system[1] Disruptions in early life microbial development have been linked to a variety of diseases including increased risk of obesity, asthma and neurodevelopmental disorders[2]. Following birth, the appearance of a diverse community of microbes takes place in a process called ecological succession[3]. Early colonisers are deemed especially influential to this succession and determine the success of subsequent colonisers[4] Environmental factors, particularly diet, are key determinants of the early colonisers and subsequent intestinal ecosystem.

Breast milk is the ideal source of nutrients, energy and bioactive compounds for newborns and is vital for postnatal growth and development[5–7]. Infant formula is the alternative to provide or complement optimal nutrition for newborns when breastfeeding is not available or is inadequate. Human milk oligosaccharides (HMO) are the third most abundant solid component (> 10 g/l) after lactose (> 60 g/l) and lipids (> 30 g/l) in breast milk[8–11]. While non-digestible by host enzymes, HMO can be utilised by certain human *Bacteroides*, *Akkermansia muciniphila* and *Bifidobacterium* strains and rodent *Enterococcus gallinarum* strains, which are considered principal drivers of diet–microbe interactions in early life[8,11–14]. HMO also partially incorporate into the systemic circulation, commonly detected in the plasma and urine of breast/HMO-fortified formula-fed infants and suckling rats[15–19].

HMO profiles in breast milk, mainly fucosylated HMO, depend on the mother's glycosyltransferase *FUT2* (*se*) and *FUT3* (*Le*) genotypes[20]. Mothers with a functional *FUT2* gene allele, known as secretors, are able to synthesise $\alpha$1–2 fucosylated HMO, and those with functional *FUT3* gene allele, known as Lewis positives, secrete high content of $\alpha$1–3/-4 fucosylated HMO[20–23]. In multiple birth cohorts, the fucosylated HMO in secretors constitute up to 45–60 % of total HMO and significantly differ in concentration and composition from that of non-secretors[10,14,24–27]. The overall intestinal microbiota, faecal glycan profiles, and growth and clinical characteristics of the

**Abbreviations:** BW, body weight; HMO, human milk oligosaccharide; NEC, necrotising enterocolitis.

* **Corresponding author:** Raylene A. Reimer, email reimer@ucalgary.ca

infants from these cohorts, however, did not vary greatly based on their mother's secretor status[14,24,26,28]. The extensive coexistence of multiple *Bifidobacterium* species in infants' microbiota, which is generally considered as a symbiotic relationship between host and the gut microbiota obtained through long-term coevolution, likely reflects their resilience to the variable HMO profiles in mothers of varying secretor status[11,14,26,29,30].

Beyond the fucosylated HMO, the sialylated HMO count for 15–25 % of total HMO and have been shown to be significantly less abundant in the milk of Malawian mothers with severely stunted infants[10,24]. This association with growth was further verified by the administration of purified sialylated bovine milk oligosaccharides to germ-free mice and pigs colonised with a bacterial strain consortium from an undernourished Malawian infant. Supplementation was able to produce significant growth promotion and metabolic changes in liver, muscle and brain[10]. Similarly, adding purified sialylated bovine milk oligosaccharides improved bone growth in germ-free mice colonised with gut microbiota from a 6-month-old stunted infant by decreasing osteoclastgenesis while sparing osteoblast activity, actions which the fucosylated HMO 2′FL failed to elicit[31].

The Malawian study highlights the structural specificity of milk oligosaccharides and the potential implications for infant growth and development. From the perspective of long-term evolution, the fucosylated milk oligosaccharides are less likely central to human lactation due to their significant variations across populations, while the unusual presence of relatively consistent sialylation of milk oligosaccharides may be more influential in the fundamental development of newborns[32,33]. It is also worth noting that the growth-promoting effects of sialylated HMO have been observed and validated in animal models of undernutrition[10,34,35]. Although the addition of these components has the potential to bring infant formula closer to human breast milk, due to the diversity of structures and complexity of purification, before the industrialised manufacture of several HMO in 2015, no single HMO was used in commercial infant formula[9,36]. In most clinical HMO studies, the two prominent HMO, 2′FL and lacto-N-neotetraose (LNnT), have been supplemented into infant formula and resulted in similar growth (body and head circumference) to that of normal formula/breastfed infants[19,34,37].

To our knowledge, *in vivo* studies comparing the function of fucosylated and sialylated HMO are limited. To test our hypothesis that fucosylated and sialylated HMO exert different impacts on neonatal growth and microbial development, we used an artificial rearing system called the pup-in-a-cup model[38–40] to examine the effects of two HMO alone or in combination in rats from postnatal days 4 to 21. Two commercially available HMO, 2′-*O*-fucosyllactose (2′FL) and 3′-*O*-sialyllactose (3′SL) were selected as representative fucosylated and sialylated HMO.

## Materials and methods

### Preparation of rat milk substitutes

Basal rat milk substitute was prepared with ingredients listed in Table 1 and served as the control diet (CTR). Three HMO interventions were prepared with basal milk substitute and the addition of 1·2 g/l 2′-*O*-fucosyllactose (96·1 w/w%, 2′FL), or 1·2 g/l 3′-*O*-sialyllactose (97·5 w/w%, 3′SL), or 0·6 g/l 2′-*O*-fucosyllactose and 0·6 g/l 3′-*O*-sialyllactose (2'FL + 3'SL). The basal milk substitute served as an appropriate control formula that was based on mature cows' milk which has an extremely low oligosaccharide concentration (0·1 g/l) compared with mature human milk (5–15 g/l)[41]. The concentration of HMO in the present study was selected based on the average intake of total fucosylated or sialylated lactose (~500 mg/kg per d, including 2′FL; 3′FL; 3′SL; 6′SL; DFLac, difucosyl-lactose) in infants at 4–12 months of age[42,43]. Ingredients were homogenised by immersion dispersers (Kinematica AG) at a speed of 15,500 rpm/s for 10 min × 3 times to avoid delamination. The homogenised rat milk substitutes were placed in 90 ml sterile containers and stored at 4°C until used. Rat milk substitutes were prepared every 3 d following the same protocol.

### Animals and establishment of pup-in-a-cup model

Ethical approval was granted by the University of Calgary Animal Care Committee (no. AC19-0104) and followed the guidelines of the Canadian Council on Animal Care. Examination of both male and female rats is important in nutrition research but based on the complexity of establishing the pup-in-a-cup model, the limited number of 'cups' that can be placed in the heated water bath at one time and in view of recent human clinical studies showing no sex differences in growth, head circumference and gastrointestinal tolerance[19,34,37] with HMO supplementation, we examined only one sex (males) in the present study. Fifty-four male Sprague–Dawley rats at 4 d of age with an average body weight (BW) of 10–12 g were obtained from the University of Calgary Life & Environmental Science Animal Care facility and *n* 13–14 pups/group randomised to CTR, 2′FL, 3′SL and 2′FL + 3′SL. The entire artificial rearing system was placed in a humidity-controlled room maintained at 30°C with a 12-h reverse light–dark cycle.

The procedure for establishing the pup-in-a-cup model was as follows: (1) Prior to surgery, hypothermic anaesthesia was applied to pre-weighed pups; (2) A cannula made of polyethylene (PE)10 tubing was pierced through the lining of the cheek and reinforced in place (Fig. 1(a)); (3) After cannulation, all pups remained on a heating blanket until warmed to normal body temperature and were then individually placed in a pre-heated and labelled foam cup (11 cm diameter × 15 cm deep). The temperature inside the cups was maintained at 34–37°C during the first week and then gradually reduced by 2–3°C/week; (4) The cheek cannula was connected to a multi-channel syringe pump (NE-1200 Twelve Channel Syringe Pump, Bio-Lynx Scientific Equipment Inc.) programmed for the delivery of the milk substitutes. Cannulated pups were randomly allocated to one of four rat milk substitutes (CTR, 2′FL, 3′SL, 2′FL + 3′SL) that were delivered with the same flow speed adjusted based on age and daily average BW as flow speed = (0·35 + 0·02 (Age-4)) × BW/feeding hours (Fig. 1(b)) with a 15-min stop flow interval programmed for every 2 h of feeding; (5) Milk substitutes were replaced twice a day with syringe replacement and a flush of the tubing.

**Table 1.** Ingredients found in the rat milk substitute

| Ingredient | Concentration(/100 ml) | Supplier | Identifier |
|---|---|---|---|
| Milk base | | | |
| Evaporated milk* | 75 ml | Carnation® | https://www.carnationmilk.ca/En/Products/Carnation-Evaporated-Milk |
| Fat | | | |
| Maize oil† | 6 ml | Mazola® | https://www.mazola.com/products/mazolareg-corn-oil/ |
| Mineral solution | | | |
| $FeSO_4{\cdot}7H_2O$ | 27 mg | Sigma-Aldrich, Inc. | https://www.sigmaaldrich.com/catalog/product/sigald/215422?lang=en&region=CA |
| $CuSO_4{\cdot}5H_2O$ | 15 mg | Sigma-Aldrich Inc. | https://www.sigmaaldrich.com/catalog/product/sigma/c8027?lang=en&region=CA |
| $ZnSO_4$ $7H_2O$ | 16 mg | Sigma-Aldrich Inc. | https://www.sigmaaldrich.com/catalog/product/sigald/221376?lang=en&region=CA |
| Amino acid | | | |
| L-Methionine | 0·1 g | Dyets Inc. | https://dyets.com/ingredients/ |
| L-Tryptophan | 0·05 g | Dyets Inc. | https://dyets.com/ingredients/ |
| Vitamin mix | | | |
| AIN-93VX‡ | 1·5 g | Dyets Inc. | https://dyets.com/vitamin-mix-compositions/ |
| Sterile water | 17 ml | NA | NA |
| Energy density | 152 kcal (636 kJ) | | |

* Carnation evaporated milk provided (per 100 g) 134 kcal; saturated fat 4·6 g, polyunsaturated fat 0·2 g, monounsaturated fat 2·3 g; cholesterol 29 mg; Na 106 mg; K 303 mg; total carbohydrate 10 g and protein 7 g, 3′SL < 20 mg, 6′SL < 2 mg, 2′FL < 1 mg. SL, sialyllactose, FL, fucosyllactose.
† Mazola maize oil provided (per 14 g) 120 kcal; saturated fat 2 g, polyunsaturated fat 7 g, monounsaturated fat 4 g and vitamin E 2 mg.
‡ Vitamin mixture AIN-93VX provided (per 1 g) thiamin HCl 6 mg, riboflavin 6 mg, pyridoxine HCl 7 mg, niacin 180 mg, Ca 16 mg, folic acid 2 mg, biotin 0·2 g, cyanocobalamin (vitamin $B_{12}$) 25 g, vitamin A palmitate 4000 μg, vitamin E acetate 75 μg, vitamin $D_3$ 1000 μg and vitamin $K_1$ 0·75 mg.

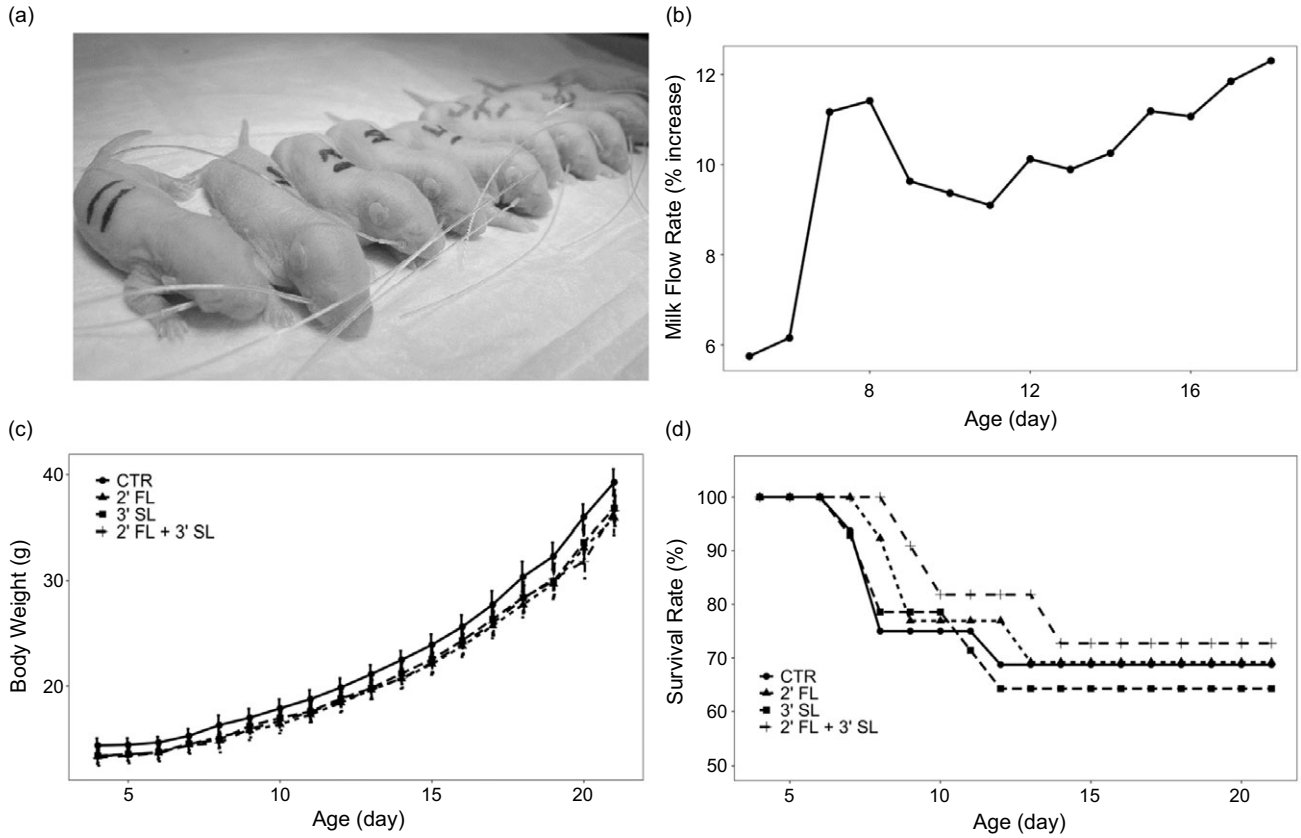


**Fig. 1.** Establishment of the pup-in-a-cup model. Pups at postnatal day 4 with cheek cannula in place (a), milk flow rate (% increase) (b), growth curve (c) and survival rate (d) of pups during 18 d of artificial rearing. No significant differences in daily body weight were observed between HMO interventions. Milk flow rate (% increase) = (flow speed at age $_{(n)}$ − flow speed at age $_{(n-1)}$)/ flow speed at age $_{(n-1)}$ × 100. Survival rate (%) = survival pup numbers/total pup number per diet group × 100. HMO, human milk oligosaccharide.

### *Daily monitoring, sample collection and measurements*

Pups were weighed twice a day throughout the 18 d of artificial rearing (Fig. 1(c)). Bowel movements and food intake behaviours were monitored daily. A dose of 0·1–0·2 ml deoxycholate solution (0·5 g/100 ml) was given to the pups if abdominal bloating occurred. The pups were euthanised if the bloating had not subsided after 48 h of treatment. Three faecal samples (week (W)1, W2 and W3) for each pup were collected by


British Journal of Nutrition

homogenising the faeces collected at 5–7, 12–14, 19–21 d of age. Pups underwent an oral glucose tolerance test at 18 d of age, as previously described[44]. The fat mass and lean mass of pups at weaning were measured using dual-energy X-ray absorptiometry (Hologic ODR 4500; Hologic Inc.) under light anaesthetisation with isoflurane. Following a 3 h fast, animals were euthanised by over anaesthetisation and rapid decapitation. Blood, intestinal tissues and caecal digesta samples were collected, and the brain, liver and caecum weighed.

### *DNA extraction and gut bacterial community profiling*

To evaluate the effects of HMO interventions on the development of faecal microbiota of pups throughout the 18 d of artificial rearing, total bacterial DNA was isolated from the W1, W2 and W3 faecal samples of thirty-six weanling pups ($n$ 108). Prior to DNA extraction using FastDNA spin kit (MP Biomedicals), faecal samples were pre-treated with bead-beating (MP Biomedicals) for 40 s × 3 times. Purified DNA was quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen) and diluted to 20 ng/μl for use.

The V3–V4 region of the16S rRNA gene was sequenced on 2 × 300 bp Illumina MiSeq platform (Centre for Health Genomics and Informatics) and analysed in QIIME2 platform using DADA2 for sequences quality control and denoising[45,46] (QIIME2 2020·11). Only the amplicon sequence variants with a frequency greater than 10 were retained for downstream analysis. The taxonomy of amplicon sequence variants was assigned by aligning to Silva 138 reference database. Amplicon sequence variants classified as genus *Lactobacillus* were filtered out and additionally aligned to Genome Taxonomy Database as references release 95 (GTDB, https://gtdb.ecogenomic.org/) to reflect the current taxonomy of *Lactobacillaceae*[47]. Additional quantitative PCR assays were conducted to quantify the abundance of total bacteria using a universal bacterial primer (forward: 5′-CGGCAACGAGCGCAACCC-3′ and reverse: 5′-CCATTGTAG CACGTGTGTAGCC-3′) targeting the conservation region of 16S rRNA gene[48,49] and *E. gallinarum* species using a primer (forward: 5′-TTACTTGCTGATTTTGATTCG-3′ and reverse: 5′-TGAATTCTTCTTTGAAATCAG-3′) targeting the *E. gallinarum*-specific region of gene *sodA*, as previously described[12,50]. Amplicon sequence was validated on Sanger Sequencing Economy platform (Centre for Health Genomics and Informatics).

### *Statistical analyses*

Outcomes with multiple measurements over time were assessed using repeated-measures ANOVA in R version 4.0.0 (2020-05-18). Body composition, total bacteria abundance and organ weight at weaning and the AUC values from the 2-h oral glucose tolerance test were analysed using linear mixed-effects models in R version 4.0.0 (2020-05-18). Dietary treatment was treated as a fixed factor; rats were considered as experimental units and their random effect was removed. Diet and/or age effects on the relative abundance of 16S rRNA gene sequence variants and weighted UniFrac distances of faecal microbiota were analysed using the Kruskal–Wallis rank-sum test in R version 4.0.0 (2020-05-18). All data are presented as mean values with their standard error of the mean. $P$ values with Bonferroni-adjustment < 0·05 were considered significant. To determine the adequate sample size to identify significant differences in the relative abundance of bacterial genera in the faecal matter of the pups, a sample size calculation utilising a power of 0·80 and $\alpha$ probability of 0·05 was performed using https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html. The effect size was estimated using the results from a previous study[44]. From the power analysis, it was determined that thirty-six rats ($n$ 9/group) were needed to complete the study.

## Results

### *Establishment of the pup-in-a-cup model*

To formulate a rat milk substitute that is close to rat's milk in macronutrients and energy, the basal rat milk substitutes used in the present study contained 12 g fat, 5·25 g protein and 7·5 g total carbohydrates. The calculated energetic density was 6359·68 kJ/l (152 kcal/100 ml), which is comparable with that of actual rat milk and the previously described formula for neonate rats 6485–8452 kJ/l (155–202 kcal/100 ml)[10,51,52]. The energetic content of the other three rat milk substitutes was considered the same as the basal milk substitute, as the 1·2 g/l HMO-related energy difference is as low as 19·6–20·4 kJ/l[53]. The total volume of milk substitutes delivered increased from 4·75 (SEM 0·12) ml/d at 4 d of age to 25·25 (SEM 0·51) ml/d at weaning, corresponding to an increase in energetic intake from 30·17 (SEM 0·75) kJ/d (7·21 (SEM 0·18) kcal/d) to 160·58 (SEM 3·26) kJ/d (38·38 (SEM 0·78) kcal/d). A total of thirty-nine of fifty-four (72·2 %) pups survived until weaning (Fig. 1(d)) which did not differ according to treatment ($P$ > 0·05). The average BW of pups increased from 13·56 (SEM 0·34) g to 37·14 (SEM 0·75) g (Fig. 1(b)). All the surviving pups opened their eyes at 12–14 d of age. The highest mortality was observed at 8–12 d of age (Fig. 1(d)). No dietary differences were observed in the age at which eyes opened or mortality ($P$ > 0·05; Fig. 1(d)).

### *Effect of human milk oligosaccharides on physical outcomes of artificially reared suckling pups*

To determine whether HMO differentially affected the growth and physiological development of artificially reared newborn rats, Lee index (Fig. 2(a1)), body composition (Fig. 2(a2,a3)) and organ weights (Fig. 2(b1–3)) at weaning, average daily weight gain (Fig. 2(c1)), as well as glucose tolerance (Fig. 2(c2,c3)) at 18 d of age were measured. The Lee index (weight $^{0·33}$/naso-anal length × 1000) of weanling pups[54] did not differ between groups ($P$ > 0·05). Mean weanling fat mass (%) and lean mass (%) reached 9·71 (SEM 2·75) % and 72·49 (SEM 6·38) %, respectively, and did not differ between groups ($P$ > 0·05). At weaning, the brain, the liver and the caecum weighed 1·31 (SEM 0·13) g, 1·49 (SEM 0·23) g and 0·18 (SEM 0·05) g, respectively, and did not differ by group ($P$ > 0·05). The pups gained 0·37 (SEM 0·03) g/d during the first 3 d (postnatal days 4–7) of artificial rearing and the rate of growth increased to 1·00 (SEM 0·20) g/d and 2·4 (SEM 0·28) g/d in days 8–14 and 15–21, respectively. Blood glucose concentrations were significantly affected by time (Fig. 2(c2), $P$ = 0·001) but not affected by HMO interventions ($P$ = 0·99), or the interaction of time × HMO interventions

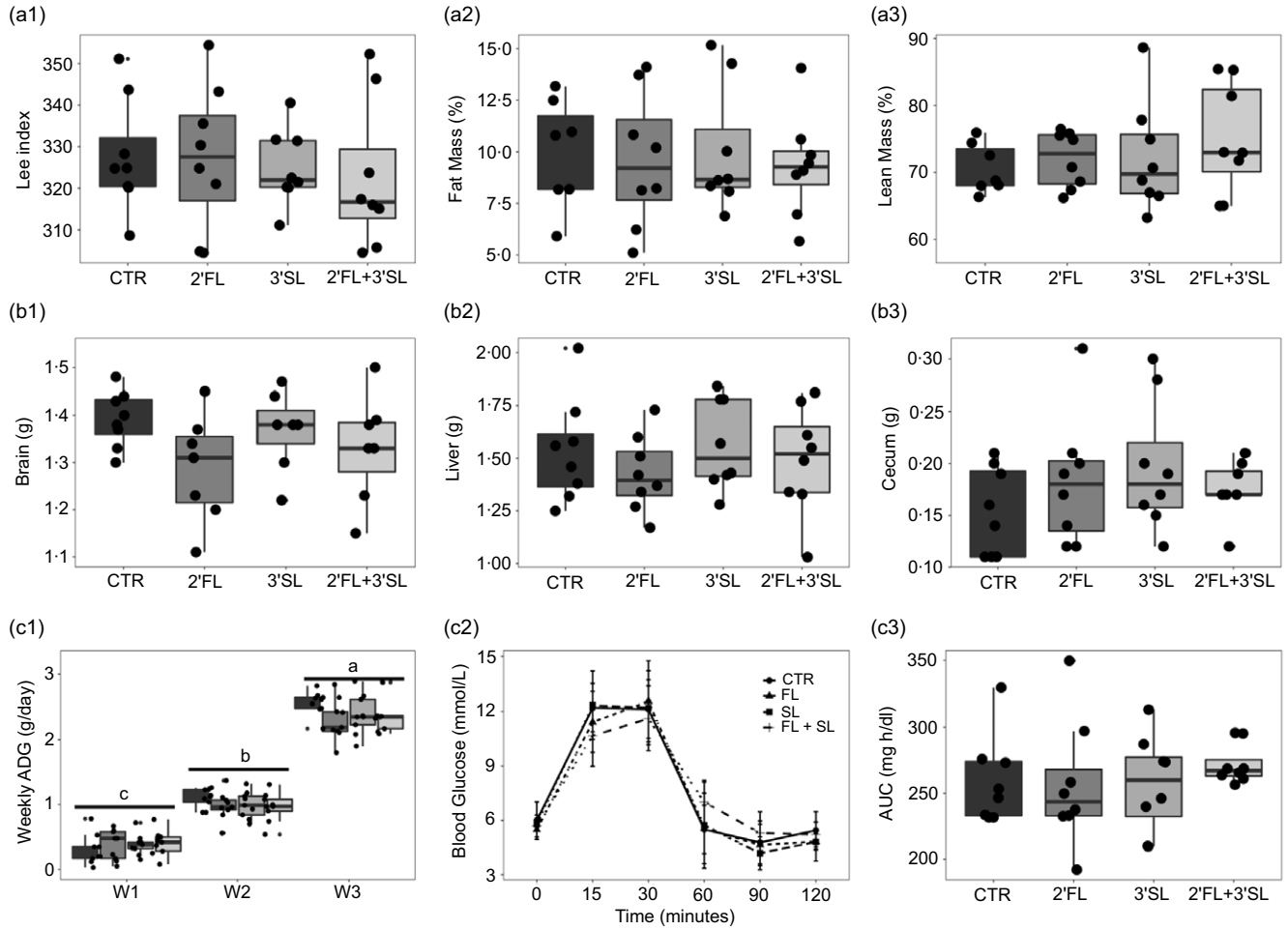


**Fig. 2.** Effect of HMO interventions on physical outcomes in artificially reared suckling rats. Difference in Lee index (a1), fat mass % (a2), lean mass % (a3), brain weight (b1), liver weight (b2), caecum weight (b3), average daily weight gain (c1), glucose tolerance (c2) and glucose AUC (c3) in suckling rats reared with CTR: basal rat milk substitute; 2′FL: CTR + 1·2 g/l 2′-fucosyllactose; 3′SL: CTR + 1·2 g/l 3′-sialyllactose; 2′FL + 3′SL: CTR + 0·6 g/l 2′-fucosyllactose + 0·6 g/L 3′-sialyllactose. Data with different superscripts represent significant difference ($P < 0·05$). Lee index = (weight $^{0·33}$/length) × 1000 (g $^{0·33}$/cm). HMO, human milk oligosaccharide.

($P = 0·48$). No dietary difference was observed in the total AUC (Fig. 2(c3)) for the 2-h oral glucose tolerance test, growth performance and physical outcomes described above ($P > 0·05$).

### *Stepwise development of gut microbiota in artificially reared pups*

The inclusion of 1·2 g/l HMO in the rat milk substitute did not influence the structure of faecal bacterial communities (online Supplementary Table S1, Fig. 3(a), $R = –0·01$, $P = 0·479$). However, across the 18 d of artificial rearing with rat milk substitutes, the development of pups' faecal microbiota was governed by age and demonstrated a stepwise pattern (online Supplementary Table S1, Fig. 3(b), $R = 0·33$, $P = 0·001$, Fig. 3(d)). The weighted UniFrac distances of W1, W2 and W3 faecal samples, which depict the dissimilarities of microbial community structure, were significantly different from each other (online Supplementary Table S1, Fig. 3(b); $P > 0·05$). The richness (observed features) and evenness did not differ between W1 and W2 faecal samples but were significantly increased in W3 faecal samples ($P = 0·0001$; online Supplementary Table S1). The total bacteria, quantified as Log10 copy number of 16S rRNA gene ng$^{-1}$ genomic DNA using qPCR, were observed to be present at a consistent level across groups and did not differ significantly between time points ($P = 0·284$) or dietary treatments ($P = 0·156$) (mean overall = CTR, 7·79 (SEM 0·09); 2′FL, 7·79 (SEM 0·08); 3′SL, 7·91 (SEM 0·11); 2′FL + 3′SL, 7·56 (SEM 0·09)). *E. gallinarum*, recently shown to be a degrader of 3′SL in suckling rats[12,50], was only detected in four out of eighty faecal samples assessed by qPCR (data not shown).

The inclusion of 1·2 g/l 2′FL did not influence the composition of faecal microbiota in artificially reared suckling rats (Fig. 3(c), $P > 0·05$). However, the presence of 3′SL (3′SL or 2′FL + 3′SL) in rat milk substitutes significantly reduced the relative abundance of genus *Proteus* in W1 faecal samples (Fig. 4(a), online Supplementary Table S2, $P = 0·009$) and genus *Terrisporobacter* in W3 faecal samples (Fig. 4(b), online Supplementary Table S2, $P = 0·008$). The relative abundance of twenty out of the thirty most abundant faecal bacterial genera of artificially reared suckling rats significantly changed ($P < 0·05$) over time (online Supplementary Table S2). The ternary plots

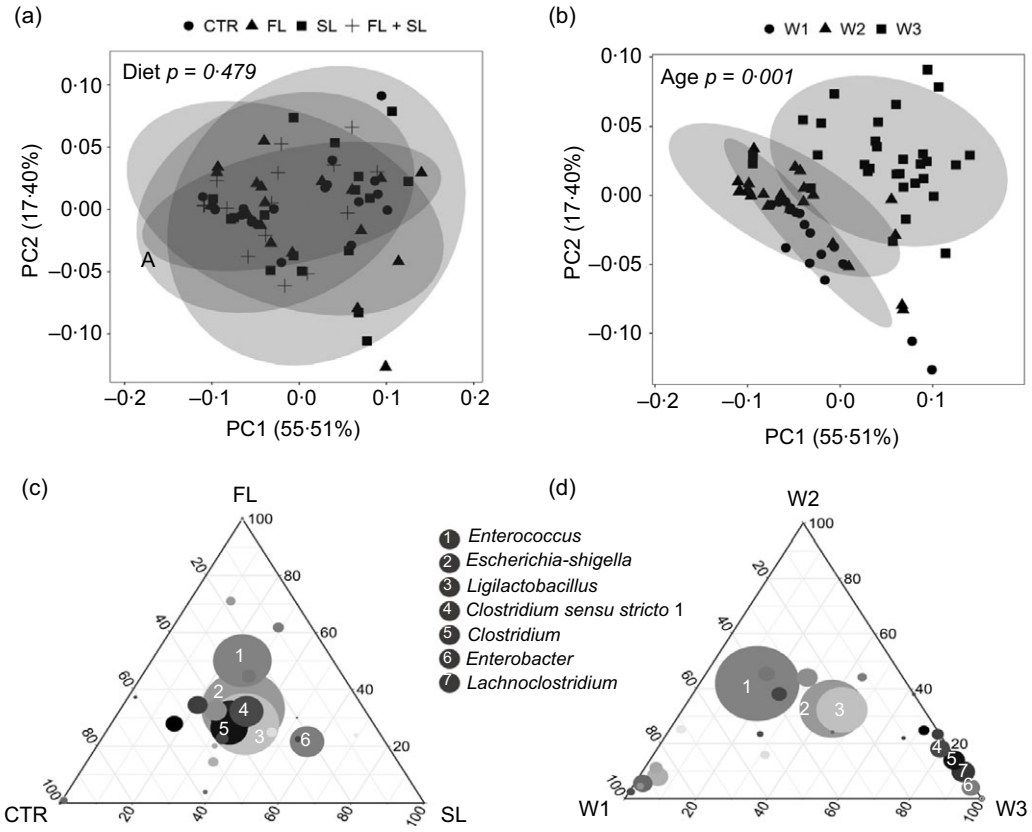


**Fig. 3.** Influence of HMO interventions and age on faecal microbiota of artificially reared suckling rats. Principal coordinate analysis (PCoA) (a), (b) and ternary plots (c), (d) demonstrating the structural and compositional differences in faecal microbiota of suckling rats fed with different HMO (a), (c) or at different ages (b), (d). CTR, basal rat milk substitute; 2′FL, CTR + 1·2 g/l 2′-fucosyllactose; 3′SL, CTR + 1·2 g/l 3′-sialyllactose; 2′FL + 3′SL, CTR + 0·6 g/l 2′-fucosyllactose + 0·6 g/l 3′-sialyllactose. HMO, human milk oligosaccharide.

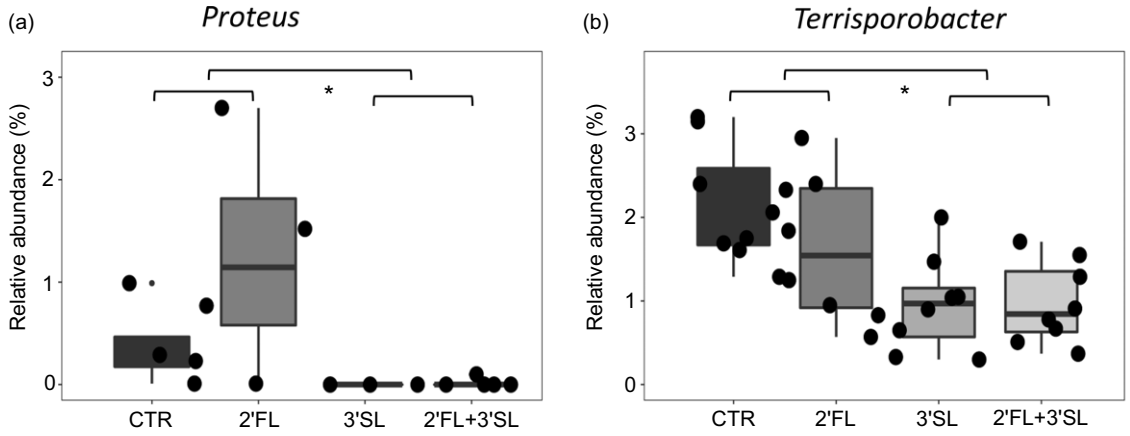


**Fig. 4.** Inclusion of 3′SL reduced select bacterial genera in the faecal microbiota of artificially reared suckling rats. Relative abundance (%) of *Proteus* in week 1 (W1) samples (a) and *Terrisporobacter* in week 3 (W3) samples (b) in faecal microbiota of artificially reared suckling rats. Data with asterisk (*) are significantly different ($P < 0·05$) between diet groups. CTR, basal rat milk substitute; 2′FL, CTR + 1·2 g/l 2′-fucosyllactose; 3′SL, CTR + 1·2 g/l 3′-sialyllactose; 2′FL + 3′SL, CTR + 0·6 g/l 2′-fucosyllactose + 0·6 g/l 3′-sialyllactose.

(Fig. 3(d)) and boxplots (Fig. 5(a1)–(c3)) depicted the abundant changes of the representative genera. Specifically, in W3 samples, the abundance of *Enterococcus* (Fig. 5(a1)), which was the most abundant genus in W1 and W2 samples (> 50 %), decreased significantly ($P = 0·0001$). Conversely, the relative abundance of *Escherichia-Shigella, Ligilactobacillus*, *Blautia*, *Lachnoclostridium*, *Clostridium*, *Clostridium* sensu stricto 1, *Terrisporobacter* and *Proteus* in W3 samples was significantly ($P < 0·05$) higher than that in W1 and W2 faecal samples (Fig. 5(a2)–(c3), online Supplementary Table S2).

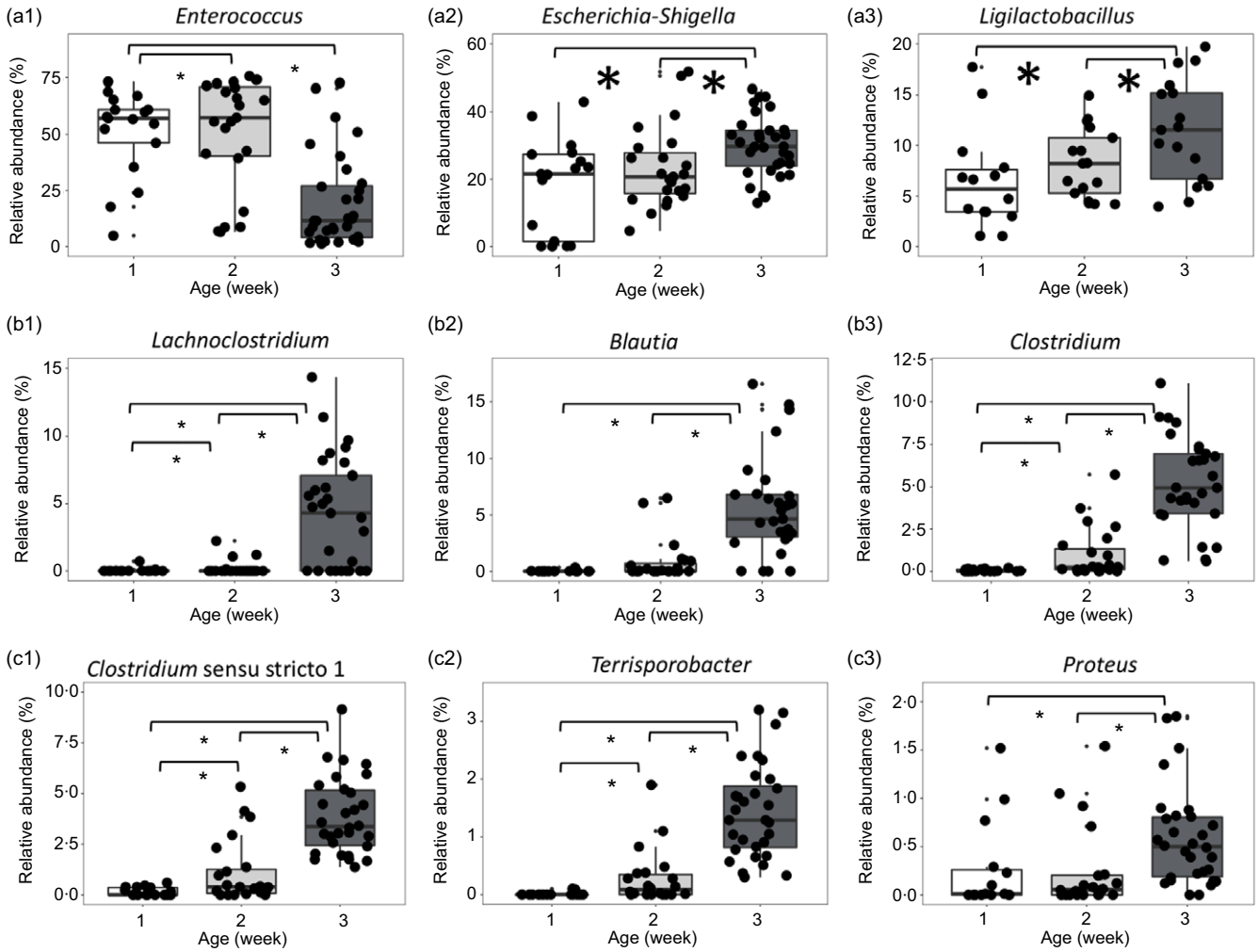


**Fig. 5.** Bacterial genera showing significant differences in faecal microbiota of artificially reared suckling rats. Data with asterisk (*) are significantly different ($P < 0{\cdot}05$) between age groups.

## Discussion

In the present study, we established an artificial rearing system to examine the effects of select HMO during the suckling period. We selected the most abundant fucosylated HMO, 2′FL and one of the abundant sialyllactose that typically remains stable during lactation, 3′SL to determine if supplementation with physiological doses of these HMO affected growth and microbial outcomes in rat pups over an 18-d investigative period[55–58]. With the exception of the potential pathogen inhibitory effects of 3′SL, no other distinct differences in growth performance and physiological outcomes were observed in the rats. Longitudinal changes in the faecal microbiota of artificially reared suckling rats were primarily governed by age and not affected by the presence of 2′-FL and/or 3′-SL in rat milk substitutes.

The pup-in-a-cup model allowed us to assess whether the addition of two HMO to the control milk substitute, meant to imitate human infants solely fed with infant formula, had any impacts on developmental parameters. Although it is challenging to assess the average milk intake of dam-fed suckling rats, we formulated a milk substitute that is close to rat milk in macronutrient content and energy density and increased the flow rate with age and BW until weaning[10,51,52]. Compared with inducing overnutrition or undernutrition in suckling rats by adjusting litter size at birth, the established artificial rearing system herein is capable of precisely controlling the nutritional supply and thus the nutritional status of newborns[59,60]. The cheek cannula we adopted is less invasive and movement restricting than the intragastric cannula used in a previous pup-in-a-cup rodent model[61]. However, feeding with a uniform flow rate via the cheek cannula is also a leading cause of death during the age of 6–13 d, the critical stage for structural and functional changes in lung development of newborn rats[62,63]. Rat pups with poor adaptability to increasing flow rate (needed to maintain proper weight gain and growth) were prone to die from choking on the milk and suffocation when the flow rate increased sharply after the age of 8 d. In addition to selecting pups with similar BW, further improvement is needed in the model to fulfil the nutritional requirement of pups while reducing choking-associated mortality. More importantly, single-cup feeding enables the random

allocation of dietary treatments within and between litters and eliminates the interference of litter effects on neonatal gut microbiota[64,65]; therefore, the pups instead of the litters can be treated as experimental units, which greatly reduces the use of animals.

Neonatal rats are born with an immature gut with respect to the stage of functional and immunological development, which develops slowly during early and mid-lactation and matures rapidly around weaning[38,66]. The general stepwise pattern of gut microbiota observed in normally suckled and now our artificially reared suckling rats consistently demonstrates these developmental milestones[12,64,65,67]. The shared nature of the microbial shifts between breastfed and our artificially reared rats also points towards a similar developmental physiology of the intestine, particularly, the dramatic shift in the luminal environment towards anaerobic conditions around weaning. In normal suckled pups, the abundance of *E. gallinarum*, the major SL-degrading species recently identified in rats, markedly increases from 7 to 12 d and decreases thereafter, indicating a critical period of colonisation with this species[12,67]. *E. gallinarum*, however, was absent in most of our pups, which were separated from their mothers at 4 d of age, indicating a possible interruption of the maternal–offspring microbial transmission. In addition to the distinct milk oligosaccharide-degrading bacterial consortium in infants (*Bacteroides*, *Akkermansia muciniphila* and *Bifidobacterium*) and suckling rats (*E. gallinarum*), the increase in strict anaerobes in exclusively breastfed infants, represented by HMO-degrading bifidobacteria, starts from the first days of life and dominates the gut microbiota from the first weeks of life[12,67–69]. This high bifidobacteria abundance was not seen in our artificially reared rats, revealing a divergent developmental manner in intestinal physiology[12,67–69]. These newfound differences identified by our pup-in-a-cup model underscore the need to further probe the limitations of the rat model for human-directed HMO microbiome research.

Despite differences in dominant HMO-degrading bacteria in rats and humans, the findings of a series of necrotising enterocolitis (NEC) studies in preterm infants and neonatal rats may shed some additional insights into the benefits of HMO[51,70–75]. A lower risk of NEC has consistently been observed in breastfed preterm infants compared with their formula-fed counterparts[73–75]. A neonatal rat NEC model has consequently been used to explore the reasons for this differential risk, which identified HMO, specifically, disialyllacto-*N*-tetraose as the determinant of the NEC-protective effects of mother's milk[73]. Two NEC-disialyllacto-*N*-tetraose clinical studies subsequently confirmed disialyllacto-*N*-tetraose deficits in the milk of mothers of infants who developed NEC and determined that a threshold level of 241 nmol/ml disialyllacto-*N*-tetraose in mother's milk could be a potential biomarker to predict the risk of NEC development in preterm infants[74,75]. Notably, associations between the relative abundance of *Bifidobacterium longum* and NEC were also shown in one of the two NEC cohort studies[75]. Even though the mechanism of the NEC-protective effects of HMO is not yet fully understood, these results collectively imply a possible existence of a mechanism in humans and rats that is not mediated by a common HMO-degrading microbiome as a core factor. Instead, the presence of most HMO structures in serum and their excretion in urine could suggest a possible association between the systemic absorption of HMO and their general protective effects in infants and suckling rats[15–19]. Importantly, the reduced abundance of the potential pathogens, *Proteus* and *Terrisporobacter*, following 3′SL ingestion by our rats could be seen as protective given that *Proteus* has been associated with Crohn's disease and inflammation[76] and *Terrisporobacter* has been linked to oxidative stress and inflammation in preterm infants fed formula *v.* human milk[77]. Even with the lower dose of 3′SL in the combination treatment (0·6 g/l compared with 1·2 g/l in the 3′SL alone group), the inhibitory effect on *Proteus* and *Terrisporobacter* was still present. This might be attributed to the previously shown ability of 3′SL exposure to cause Caco-2 cells to change their surface glycan profile[78,79]. It is possible that even the lower dose in our combination treatment was able to modify the glycan content on the surface of the rats' epithelial cells and receptor sites for these potential pathogens.

Compared with human and rat milk, mature bovine milk contains almost no non-fucosylated oligosaccharides and a significantly lower content of 3′SL (0·04–0·12 g/l)[33,80,81]. The basal rat milk substitute we used was formulated based on evaporated bovine milk, which would consequently mimic the 2′FL and 3′SL deficits in our artificially reared pups. Overall, when supplementing with these two HMO, we observed the potential pathogen inhibitory effect of 3′SL but did not identify any differences in growth performance, body composition and organ weights in pups fed with 2′FL or 3′SL milk replacer. Although this lack of difference in growth is similar to human studies with HMO supplementation[19,34,37] that included both male and female infants, we only examined male rats and it would be important to repeat this work with female rat pups. Given previous research[74], it is possible that the HMO could have affected immune response without affecting growth and the other parameters we examined; however, we did not assess any immune parameters. Furthermore, the reduction in potential pathobionts such as *Terrisporobacter*, while important in healthy normally developing infants, could be even more relevant in preterm or undernourished infants. It would be important in future studies to examine a broader scope of metabolic and immunologic outcomes[10]. Given that LNnT, difucosyllactose (DFL), lacto-N-tetraose (LNT) and 6′SL can now be produced on a commercial large scale[9], using additional HMO structures in future studies would be helpful in identifying whether there are more structure-specific attributes of HMO. In addition, given the relevance between the protective effects of sialylated HMO and host abnormalities (premature birth, extremely low BW or malnutrition)[10,73–75], further investigation into the protective effects of specific HMO on the host under abnormal conditions may have greater clinical significance in neonatal research.

## Acknowledgements

The authors wish to thank Shelly Wegener and Dr. Richard Pon, Centre for Health Genomics and Informatics at the University of Calgary, for support with the 16S rRNA sequencing.

This work was supported by a research grant from the Natural Sciences and Engineering Research Council of Canada

(NSERC RGPIN/03773-2016). W. W. is supported by a University of Calgary Eyes High Postdoctoral Fellowship.

W. W. designed research, performed animal experiments, prepared samples, performed bioinformatics analysis and wrote paper. C. M. and J. S. helped with the pup-in-a-cup model and provided resources. N. A. C., E. W. N. T., D. E. L., F. C., K. M. S. and K. S. assisted with animal experiments. R. A. R. designed research, assisted with interpretation, obtained funding and had primary responsibility for final content. All authors read and approved the final manuscript.

The authors declare that they have no competing interests.

## Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114521005146

## References

1. Ratsika A, Codagnone MC, O'mahony S, *et al.* (2021) Priming for life: early life nutrition and the microbiota-gut-brain axis. *Nutrients* **13**, 423.
2. Sarkar A, Yoo JY, Dutra SVO, *et al.* (2021) The association between early-life gut microbiota and long-term health and diseases. *J Clin Med* **10**, 459.
3. Bäckhed F, Roswall J, Peng Y, *et al.* (2015) Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* **17**, 690–703.
4. Laforest-Lapointe I & Arrieta MC (2017) Patterns of early-life gut microbial colonization during human immune development: an ecological perspective. *Front Immunol* **8**, 788.
5. Kelly D & Coutts AGP (2000) Early nutrition and the development of immune function in the neonate. *Proc Nutr Soc* **59**, 177–185.
6. Harding JE, Cormack BE, Alexander T, *et al.* (2017) Advances in nutrition of the newborn infant. *Lancet* **389**, 1660–1668.
7. Butte NF, Garza C, Smith B, *et al.* (1984) Human milk intake breast-fed infants and growth in exclusively. *J Pediatr* **104**, 187–195.
8. Zivkovic AM, German JB, Lebrilla CB, *et al.* (2011) Human milk glycobiome and its impact on the infant gastrointestinal microbiota. *Proc Natl Acad Sci USA* **108**, 4653–4658.
9. Bych K, Mikš MH, Johanson T, *et al.* (2019) Production of HMOs using microbial hosts – from cell engineering to large scale production. *Curr Opin Biotechnol* **56**, 130–137.
10. Charbonneau MR, O'Donnell D, Blanton LV, *et al.* (2016) Sialylated milk oligosaccharides promote microbiota-dependent growth in models of infant undernutrition. *Cell* **164**, 859–871.
11. Lawson MAE, O'Neill IJ, Kujawska M, *et al.* (2020) Breast milk–derived human milk oligosaccharides promote Bifidobacterium interactions within a single ecosystem. *ISME J* **14**, 635–648.
12. Akazawa H, Tsujikawa Y, Fukuda I, *et al.* (2021) Isolation and identification of milk oligosaccharide-degrading bacteria from the intestinal contents of suckling rats. *Biosci Microbiota Food Heal* **40**, 27–32.
13. Kostopoulos I, Elzinga J, Ottman N, *et al.* (2020) Akkermansia muciniphila uses human milk oligosaccharides to thrive in the early life conditions *in vitro*. *Sci Rep* **10**, 1–17.
14. Lewis ZT, Totten SM, Smilowitz JT, *et al.* (2015) Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants. *Microbiome* **3**, 13.
15. Vazquez E, Santos-Fandila A, Buck R, *et al.* (2017) Major human milk oligosaccharides are absorbed into the systemic circulation after oral administration in rats. *Br J Nutr* **117**, 237–247.
16. Goehring KC, Kennedy AD, Prieto PA, *et al.* (2014) Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. *PLOS ONE* **9**, e101692.
17. Rudloff S, Pohlentz G, Borsch C, *et al.* (2012) Urinary excretion of *in vivo* 13C-labelled milk oligosaccharides in breastfed infants. *Br J Nutr* **107**, 957–963.
18. Underwood MA, Gaerlan S, De Leoz MLA, *et al.* (2015) Human milk oligosaccharides in premature infants: absorption, excretion, and influence on the intestinal microbiota. *Pediatr Res* **78**, 670–677.
19. Marriage BJ, Buck RH, Goehring KC, *et al.* (2015) Infants fed a lower calorie formula with 2 ′ FL show growth and 2 ′ FL uptake like breast-fed infants. *J Pediatr Gastroenterol Nutr* **61**, 649–658.
20. Totten SM, Zivkovic AM, Wu S, *et al.* (2012) Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers. *J Proteome Res* **11**, 6124–6133.
21. Kobata A & Yamakawa T (2010) Review structures and application of oligosaccharides in human milk. *Proc Jpn Acad Ser B* **86**, 731–747.
22. Wang A, Koleva P, du Toit E, *et al.* (2021) The milk metabolome of non-secretor and Lewis negative mothers. *Front Nutr* **7**, 576966.
23. Difilippo E, Pan F, Logtenberg M, *et al.* (2016) Milk oligosaccharide variation in sow milk and milk oligosaccharide fermentation in piglet intestine. *J Agric Food Chem* **64**, 2087–2093.
24. Liu F, Yan J, Wang X, *et al.* (2021) Maternal fucosyltransferase 2 status associates with the profiles of human milk oligosaccharides and the fecal microbiota composition of breastfed infants. *J Agric Food Chem* **69**, 3032–3043.
25. Moossavi S, Sepehri S, Robertson B, *et al.* (2019) Composition and variation of the human milk microbiota are influenced by maternal and early-life factors. *Cell Host Microbe* **25**, 324–335.
26. Moossavi S, Atakora F, Miliku K, *et al.* (2019) Integrated analysis of human milk microbiota with oligosaccharides and fatty acids in the CHILD cohort. *Front Nutr* **6**, 58.
27. Siziba LP, Mank M, Stahl B, *et al.* (2021) Human milk oligosaccharide profiles over 12 months of lactation: the ulm SPATZ health study. *Nutrients* **13**, 1973.
28. Sprenger N, Lee LY, De Castro CA, *et al.* (2017) Longitudinal change of selected human milk oligosaccharides and association to infants' growth, an observatory, single center, longitudinal cohort study. *PLOS ONE* **12**, e0171814.
29. Schell MA, Karmirantzou M, Snel B, *et al.* (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc Natl Acad Sci USA* **99**, 14422–14427.
30. Duranti S, Lugli GA, Milani C, *et al.* (2019) *Bifidobacterium bifidum* and the infant gut microbiota: an intriguing case of microbe-host co-evolution. *Environ Microbiol* **21**, 3683–3695.
31. Cowardin CA, Ahern PP, Kung VL, *et al.* (2019) Mechanisms by which sialylated milk oligosaccharides impact bone biology in a gnotobiotic mouse model of infant undernutrition. *Proc Natl Acad Sci* **116**, 11988–11996.
32. Tao N, Wu S, Kim J, *et al.* (2011) Evolutionary glycomics: characterization of milk oligosaccharides in primates. *J Proteome Res* **10**, 1548–1557.
33. Hobbs M, Jahan M, Ghorashi SA, *et al.* (2021) Current perspective of sialylated milk oligosaccharides in mammalian milk: implications for brain and gut health of newborns. *Foods* **10**, 1–19.

34. Román Riechmann E, Moreno Villares JM, Domínguez Ortega F, *et al.* (2020) Real-world study in infants fed with an infant formula with two human milk oligosaccharides. *Nutr Hosp* **37**, 698–706.
35. Chouraqui JP (2021) Does the contribution of human milk oligosaccharides to the beneficial effects of breast milk allow us to hope for an improvement in infant formulas? *Crit Rev Food Sci Nutr* **61**, 1503–1514.
36. Zhou W, Jiang H, Wang L, *et al.* (2021) Biotechnological production of 2′-fucosyllactose: a prevalent fucosylated human milk oligosaccharide. *ACS Synth Biol* **10**, 447–458.
37. Puccio G, Alliet P, Cajozzo C, *et al.* (2017) Effects of infant formula with human milk oligosaccharides on growth and morbidity: a randomized multicenter trial. *J Pediatr Gastroenterol Nutr* **64**, 624–631.
38. Puiman P & Stoll B (2008) Animal models to study neonatal nutrition in humans. *Curr Opin Clin Nutr Metab Care* **11**, 601–606.
39. Scantlebury MH, Galanopoulou AS, Chudomelova L, *et al.* (2010) A model of symptomatic infantile spasms syndrome. *Neurobiol Dis* **37**, 604–612.
40. Beierle EA, Chen MK, Hartwich JE, *et al.* (2004) Artificial rearing of mouse pups: development of a mouse pup in a cup model. *Pediatr Res* **56**, 250–255.
41. Quinn EM, O'Callaghan TF, Tobin JT, *et al.* (2020) Changes to the oligosaccharide profile of bovine milk at the onset of lactation. *Dairy* **1**, 284–296.
42. Plows JF, Berger PK, Jones RB, *et al.* (2021) Longitudinal changes in human milk oligosaccharides (HMOs) over the course of 24 months of lactation. *J Nutr* **151**, 876–882.
43. Larsson MW, Lind MV, Laursen RP, *et al.* (2019) Human milk oligosaccharide composition is associated with excessive weight gain during exclusive breastfeeding – an explorative study. *Front Pediatr* **7**, 297.
44. Chleilat F, Klancic T, Ma K, *et al.* (2020) Human milk oligosaccharide supplementation affects intestinal barrier function and microbial composition in the gastrointestinal tract of young Sprague Dawley rats. *Nutrients* **12**, 1532.
45. Bolyen E, Rideout JR, Dillon MR, *et al.* (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* **37**, 852–857.
46. Callahan BJ, McMurdie PJ, Rosen MJ, *et al.* (2016) DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Meth* **13**, 581–583.
47. Zheng J, Wittouck S, Salvetti E, *et al.* (2020) A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus Beijerinck* 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *Int J Syst Evol Microbiol* **70**, 2782–2858.
48. Denman SE & McSweeney CS (2006) Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol Ecol* **58**, 572–582.
49. Bahl MI, Bergström A & Licht TR (2012) Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. *FEMS Microbiol Lett* **329**, 193–197.
50. Jackson CR, Fedorka-Cray PJ & Barrett JB (2004) Use of a genus- and species-specific multiplex PCR for identification of enterococci. *J Clin Microbiol* **42**, 3558–3565.
51. Autran CA, Schoterman MHC, Jantscher-Krenn E, *et al.* (2016) Sialylated galacto-oligosaccharides and 2′-fucosyllactose reduce necrotising enterocolitis in neonatal rats. *Br J Nutr* **116**, 294–299.
52. Nicholas KR & Hartmann PE (1991) Milk secretion in the rat: progressive changes in milk composition during lactation and weaning and the effect of diet. *Comp Biochem Physiol – Part A Physiol* **98**, 535–542.
53. Flint HJ (2020) *Why Gut Microbes Matter: Understanding our Microbiome*. Cham: Springer.
54. Stephens DN (1980) Does the Lee obesity index measure general obesity? *Physiol Behav* **25**, 313–315.
55. Chaturvedi P, Warren CD, Altaye M, *et al.* (2001) Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology* **11**, 365–372.
56. Thurl S, Munzert M, Henker J, *et al.* (2010) Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Br J Nutr* **104**, 1261–1271.
57. Butte NF, Garza C, Smith EOB, *et al.* (1984) Human milk intake and growth in exclusively breast-fed infants. *J Pediatr* **104**, 187–195.
58. Michaelsen KF, Larsen PS, Thomsen BL, *et al.* (1994) The Copenhagen Cohort Study on Infant Nutrition and Growth: breast-milk intake, human milk macronutrient content, and influencing factors. *Am J Clin Nutr* **59**, 600–611.
59. Seidler FJ, Bell JM & Slotkin TA (1990) Undernutrition and overnutrition in the neonatal rat: long-term effects on noradrenergic pathways in brain regions. *Pediatr Res* **27**, 191–197.
60. Plagemann A, Harder T, Rake A, *et al.* (1999) Observations on the orexigenic hypothalamic neuropeptide Y-system in neonatally overfed weanling rats. *J Neuroendocrinol* **11**, 541–546.
61. Penttila IA, Flesch IEA, McCue AL, *et al.* (2003) Maternal milk regulation of cell infiltration and interleukin 18 in the intestine of suckling rat pups. *Gut* **52**, 1579–1586.
62. Roberts RJ, Weesner KM & Bucher JR (1983) Oxygen-induced alterations in lung vascular development in the newborn rat. *Pediatr Res* **17**, 368–375.
63. Bucher JR & Roberts RJ (1981) The development of the newborn rat lung in hyperoxia: a dose-response study of lung growth, maturation, and changes in antioxidant enzyme activities. *Pediatr Res* **15**, 999–1008.
64. Azagra-Boronat I, Massot-Cladera M, Mayneris-Perxachs J, *et al.* (2019) Immunomodulatory and prebiotic effects of 2′-Fucosyllactose in suckling rats. *Front Immunol* **10**, 1773.
65. Dai J, Yang X, Yuan Y, *et al.* (2020) Toxicity, gut microbiota and metabolome effects after copper exposure during early life in SD rats. *Toxicology* **433**, 152395.
66. Sangild PT (2006) Gut responses to enteral nutrition in preterm infants and animals. *Exp Biol Med* **231**, 1695–1711.
67. Matsui S, Akazawa H, Tsujikawa Y, *et al.* (2021) Milk oligosaccharide-mediated cross-feeding between Enterococcus gallinarum and lactobacilli in the gut microbiota of infant rats. *Biosci Microbiota Food Heal* **40**, 204–211.
68. Ferretti P, Pasolli E, Tett A, *et al.* (2018) Mother-to-infant microbial transmission from different body sites shapes the developing infant gut microbiome. *Cell Host Microbe* **24**, 133–145.
69. Korpela K & de Vos WM (2018) Early life colonization of the human gut: microbes matter everywhere. *Curr Opin Microbiol* **44**, 70–78.
70. Schanler RJ, Lau C, Hurst NM, *et al.* (2005) Randomized trial of donor human milk versus preterm formula as substitutes for mothers' own milk in the feeding of extremely premature infants. *Pediatrics* **116**, 400–406.
71. Sisk PM, Lovelady CA, Dillard RG, *et al.* (2007) Early human milk feeding is associated with a lower risk of necrotizing enterocolitis in very low birth weight infants. *J Perinatol* **27**, 428–433.
72. Sullivan S, Schanler RJ, Kim JH, *et al.* (2010) An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. *J Pediatr* **156**, 562–567.

73. Jantscher-Krenn E, Zherebtsov M, Nissan C, *et al.* (2012) The human milk oligosaccharide disialyllacto-*N*-tetraose prevents necrotising enterocolitis in neonatal rats. *Gut* **61**, 1417–1425.
74. Autran CA, Kellman BP, Kim JH, *et al.* (2018) Human milk oligosaccharide composition predicts risk of necrotising enterocolitis in preterm infants. *Gut* **67**, 1064–1070.
75. Masi AC, Embleton ND, Lamb CA, *et al.* (2021) Human milk oligosaccharide DSLNT and gut microbiome in preterm infants predicts necrotising enterocolitis. *Gut* **70**, 2273–2282.
76. Zhang J, Hoedt EC, Liu Q, *et al.* (2021) Elucidation of Proteus mirabilis as a key bacterium in Crohn's Disease Inflammation. *Gastroenterology* **160**, 317–330.e11.
77. Cai C, Zhang Z, Morales M, *et al.* (2019) Feeding practice influences gut microbiome composition in very low birth weight preterm infants and the association with oxidative stress: A prospective cohort study. *Free Radic Biol Med* **142**, 146–154.
78. Bode L (2015) The functional biology of human milk oligosaccharides. *Early Hum Dev* **91**, 619–622.
79. Bode L (2012) Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology* **22**, 1147–1162.
80. Tao N, DePeters EJ, German JB, *et al.* (2009) Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. *J Dairy Sci* **92**, 2991–3001.
81. Nakamura T, Kawase H, Kimura K, *et al.* (2003) Concentrations of sialyloligosaccharides in bovine colostrum and milk during the prepartum and early lactation. *J Dairy Sci* **86**, 1315–1320.


British Journal of Nutrition