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Abstract:

Oligosaccharides (OS) are commonly added to infant formulae but their physiological impacts, particularly those potentially involved in adult health programming, are poorly described. In adult animals, OS modify microbiota and stimulate colonic fermentation and enteroendocrine cells (EEC) activity. Since neonatal changes in microbiota and/or EEC density could long last and EEC-derived peptides regulate short-term food intake, we hypothesized that neonatal OS consumption could modulate early EEC with possible consequence on adult eating behavior.
Suckling rats were supplemented with FOS, GOS/inulin mix, αGOS (3.2 $g.kg^{-1}$), or control solution from postnatal days (PND) 5 to 14/15. Pups were either sacrificed at PND14/15 or weaned at PND21 to standard chow. Impacts on both microbiota and EEC were characterized at PND14/15 and eating behavior at adulthood.
Very early OS supplementation drastically impacted intestinal environment, endocrine lineage proliferation/differentiation particularly in the ileum as well as density of GLP-1 cell and production of satiety-related peptides (GLP-1 and PYY) in neonatal period but failed to induce any significant enduring changes in intestinal microbiota, enteropeptides secretion or eating behavior, later in life.
Altogether our results demonstrate no programming effect of OS on satiety peptides secreted by L-cell nor on food consumption, what is reassuring in a human perspective.

Keywords: prebiotic, gut-brain, programming, microbiota, L-cell, eating behavior

1. Introduction

Preventing unhealthy feeding behaviors is highly desirable since deleterious eating habits are associated with health problems including higher risk of overweight or obesity [Lindsay et al 2017]. Because eating behavior is the result of integrated central and peripheral biological systems that are influenced by genetic, psychologic and environmental factors [MacLean et al., 2017], its optimization is highly complex and requires to fully elucidate the mechanisms that control eating behavior. Indeed, central regulation of appetite is mediated by peripheral inputs generated by stomach distension, signals arising from gut epithelium that senses nutrient availability, such as satiety regulatory peptides synthetized and released by enteroendocrine cells (EEC), as well as long-term energy signals released by adipose tissue and cerebral inputs generated by hedonics and rewards circuits [Schwartz 2000, Berthoud et al, 2006, MacLean et al 2017].

Besides evident progress in understanding these interconnections, recent advances include two major findings: first, eating behavior may be programmed very early in life and second, it could be regulated by intestinal microbiota.

In accordance with the developmental origin of health and disease [DOHaD] theory, adverse early-life conditions may predispose to eating disorders [Gaetani 2106]. Among environmental stressors that may have an action, it is suggested from both animal and human studies that perinatal nutrition could program appetite [see for reviews Cripps et al 2005; Ross and Desai 2014; Parnet et al. 2016]. In rodents, experiments based on maternal nutrition restriction or and/or manipulation of litter size have demonstrated that both pre- and post-natal nutrition may alter food intake [Desai et al. 2007; Orozco-Solis et al 2009., Coupé et al

2011, Ndjim et al 2017] and/or food preference [Coupé et al 2012, Martin-Agnoux et al 2014; Paradis et al 2017] in offspring, with subsequent repercussions at adulthood. In humans, although controversial results have been observed about the influence of prenatal nutrition on later eating behavior [see for review van Deutekom et al. 2017], some observational evidences suggest that early nutrition/growth affects appetite regulation [Lussana et al 2008; Perala et al., 2012, van Deutekom et al. 2016] and food preference programming as demonstrated after repeated exposition to novel flavors [De Cosmi et al 2017].

With regard to the involvement of intestinal microbiota in feeding behavior, although the ability of intestinal fermentation to stimulate the expression of satiety peptides by EEC has been known for several years [Reimer and McBurney 1996, Kok et al 1998], it is only recently, in connection with the growing appreciation of the role of intestinal microbiota in the regulation of host physiology, that this topic has excited interest [van de Wouw et al. 2017; Lam et al 2017; Fetissov 2017; Glenny et al 2017].

As pointed out in these reviews, some observations objectively support the involvement of the intestinal microbiota in the regulation of feeding behavior. Thus, in ascending order of convincing power, we can quote: i) observed dysbiosis in patients suffering eating disorders [Morita et al 2015], ii) the fact that feeding behavior differs between germ-free and conventional animals [see for reviews Wostmann, 1981 or Smith et al 2007], iii) the capability of microbiota disrupting agents- particularly prebiotic oligosaccharides- to affect feeding behavior [Cani et al., 2005; Maurer et al., 2009] and, iv) the delineation of mechanistic pathways linking microbiota and central and peripheral neuroendocrine systems which are responsible for feeding behavior, what supports the existence of a causative link. For example, one can mention EEC secreting appetite-regulating peptide since they have a large diversity of receptors that allow them to integrate microbial inputs such as the fermentation derived short chain fatty acids, secondary biliary salts or the pathogens associated molecular patterns (See for review van de Wouve et al., 2017; Plovier and Cani, 2017).

Conciliating these two emerging issues related to the regulation of feeding behavior, namely its possible programming by early life and its control by intestinal microbiota, we hypothesized that early microbiota modifications may program adult feeding behavior by acting on the peripheral neuroendocrine systems that control it. Such a programming could stem from either the potential programming of intestinal microbiota [e.g.: Morel et al 2015a] or from early impacts with long lasting consequences. In this last respect, it is worth mentioning the capability of microbiota-modulating agents to affect the hypothalamic expression of neurogenic factor (BDNF) expression at neonatal stage [Williams et al 2016], and the potential programmable character of both EEC [Estienne et al 2010] and vagal sensitivity [Ndjim et al 2017]. In addition, the putative ability of the gut microbiota to modulate the epigenome [see for review Mischke and Plosch, 2013; Lallès et al 2016], as well as the ability of the microbiota [Sudo et al 2004] and of certain prebiotics [Savignac et al 2016; Burokas et al 2017] to modulate some behaviors in adults mice can be invoked, assuming the veracity/transposability of these results in the neonatal period.

To test these hypotheses, we aimed at evaluating the ability of nutritionally induced intestinal microbiota modulations to affect the maturation and functioning of EEC in the neonatal period in suckling rats and to determine whether these impacts could result in alterations in eating behavior and in GI peptides secretion at adulthood. We chose to use indigestible oligosaccharides (OS) to modify intestinal microbiota of neonatal rats for two reasons : first, OS, that are recognized as intensively fermented prebiotics [Macfarlane et al 2008] also operant in neonatal rats [Morel et al 2015a] and infants [see for review Skórka et al 2018], have been shown to stimulate enteroendocrine cells (EEC) proliferation and activity in adults animals [Delzenne et al 2007; Overduin et al 2013]; and second, they represent relevant nutrients of neonatal nutrition since they are commonly added to infant formulae to better mimic maternal milk [Sabater et al 2016].

2. Materials and Methods

Ethics statement

All experiments were conducted in accordance with the European Union regulations for the care and use of animals for experimental procedures (2010/63/EU). Protocols were approved by the local Committee on the Ethics in Animal Experiments of Pays de la Loire (France) and the French Ministry of Research (APAFIS#3652-20 160 1 1910192893 v3). Animal facility is registered by the French Veterinary Department as A44276.

Animals

Primipare female Sprague-Dawley rats (n=16) were obtained at one-day of gestation (G1) from Janvier-labs (le Genest Saint Isle, France) and housed individually (22 ± 2°C, 12:12-h light/dark cycle) with free access to water and chow (A03, Safe Diet, Augy, France). At birth, 8 litters were culled to 8 male pups per mother with systematic cross fostering as previously described [Morel et al., 2015a].

From the 5 to the 14/15th days of life, pups received by oral gavage solutions composed of FOS, GOS/In mix (9:1), αGOS or a mix of monomers that are present in the OS solutions (Table 1). Two pups of each litter received daily one of the 4 solutions. The administered volume was adapted to body weight to reach 3.2 g.kg^{-1} in order to approximate the dose actually consumed by babies fed with prebiotic enriched formulas, after accounting for both the difference in metabolic rate between rats and humans [Nair & Jacob, 2016] and the true prebiotic content of infant formula [Sabater et al 2016; Nijman et al 2018].

During lactation period, pups were weighted daily and then 3 time a week until adulthood. At postnatal day (PND) 14 or 15, 4 of the 8 litters were slaughtered by decapitation after induction of deep anesthesia (isoflurane/O_2, 5L/min). Rats from the 4 remaining litters were weaned in individual cages at PND21 onto

standard chow (A03, Safe Diet, Augy, France) until 124/126d, when they were slaughtered as described above. During the follow-up, food consumption was measured 3 times a week.

Table 1. Composition of solutions administered by gavage to pups from PND 5 to 14/15 (g/mL^{-1})

	CTL	FOS[§]	GOS*/In[†]	αGOS[$]
GOS syrup (VivinalGOS, Borculo domo)*			0.65	
Inulin powder (Raftiline HP, Orafti)[†]			0.03	
FOS powder (Beneo P95, Orafti)[§]		0.34		
αGOS powder (Olygose)[$]				0.30
α-Lactose monohydrate (Sigma, L3625)	0.096	0.096		0.096
D(+)-glucose monohydrate (Merck 108342)	0.087	0.082		0.087
D(+)-galactose monohydrate (Merck 104058)	0.005	0.005		0.004
D(-)-fructose (Sigma F0127)	0.015		0.015	0.015
Saccharose (Sigma S9378)	0.002		0.002	0.002
Total oligosaccharides[‼]		0.30	0.30	0.30
Total digestible sugars[‼]	0.20	0.20	0.20	0.20

CTL, control; FOS, fructo-oligosaccharides; GOS/In, mix of galacto-oligosaccharides and long chain fructo-oligosaccharides (In, inuline; 9:1)

** 75% dry matter composed of 59% oligomers and 41% monomers.*

† 97% dry matter composed of 99.5% oligomers.

§ 93.2% dry matter composed of 90.4% oligomers and 6.6% monomers.

$ 95.9 % dry matter composed of 95.4% oligomers.

‼ These calculations take into account the dry matter of the components, their purity, and the amount of digestible sugars they contain.

Tissue collection

Under anesthesia, intracardiac blood was collected in EDTA-containing tube (Microtubes 1.3mL K3E, Sarstedt MG & Co, Marnay, France) and plasma collected after centrifugation (2000g, 15min, 4°C) was frozen at -20°C for further analyses. Ileal contents from the most distal 15cm were harvested by flushing, using 1 mL of Hanks' Balanced Salt Solution (HBSS, Thermo Fisher Scientific, St-Herblain, France) and cecocolonic (PND 14/15) or cecal (PND124/126) content was collected, weighted, mixed in 5 or 2 (PND 14/15 and PND124/126, respectively) fold their volume with sterile water. After complete homogeneisation, these cecocolonic/cecal suspensions were centrifuged (7800g, 20min, 4°C) then both supernatants and pellets were frozen at -20°C for short chain fatty acid (SCFA) and microbiota analyses, respectively. Intestinal tissues (ileum and proximal colon) were rapidly collected and frozen in liquid nitrogen for RNA analysis. Additional tissues samples were fixed in 4% paraformaldehyde for immunofluorescence analysis.

Eating behavior

- Meal pattern

Between PND74 and PND99, eating behavior was analyzed in physiological cages (Phecomb cages, Bioseb, Vitrol, France) as previously described [Coupé et al., 2011]. Briefly, rats were housed individually and following 24 h of acclimatization to the cage, data were recorded from the beginning of the second day (8:00 a.m.) each 5 s over a 24-h period. Meal parameters extracted from Compulse v1.1.01 software included number of meals, meal size and duration, inter-meal interval, and satiety ratio.

- Taste preference

Preference for sweet taste was measured at PND110 using the bottle test experiment [Silva 1977]. After a 2d habituation to the presence of 2 bottles on their own cage, animals had the choice between 2 bottles, one containing tap-water and a second one containing 0.05% saccharin. For 3 days, drink intake was measured daily. The position of the two bottles was daily inverted to prevent position preference bias. The sweet preference score was calculated as the ratio between the volume of saccharin solution consumed and the total drink intake in 24h then multiplied by 100. A preference is defined if the percentage is superior to 50.

Kinetics of GLP-1 and PPY release and response to glucose

At PND105, a 4h-kinetics of GLP-1 and PYY release in plasma was performed. Rats were fasted during 16h to promote hunger and were refed during 20 min with a calibrated quantity of chow (A03, safe Diet). Food intake was weighted at the end of the 20min-period. Blood samples were collected at the tail vein in EDTA-containing tubes (Microvette CB300 EDTA 3K, Sarstedt, Marnay, France) at 0 (15 min before refeeding), 30, 60, 120, and 180 min after the beginning of the meal.

At PND124/126, rats were fasted during 16h and 2h before sacrifice they received an oral bolus of glucose (2 kg/kg^{-1} BW) in order to challenge glucose sensing in GLP-1/PYY-producing EEC.

Plasma gastrointestinal peptides

Plasma concentration of total GLP-1 and total PYY was assayed by ELISA technique (Millipore EIA kits and Phoenix Pharmaceutical, France, respectively).

Fermentation end-products

Ileal and cecal supernatants were centrifuged (8000 g, 20 min, 4°C), diluted (1/10) with oxalic acid 0.5 M and SCFA (acetate, propionate, butyrate, isobutyrate, valerate and isovalerate) were analysed by gas chromatography as previously described [Fança-Berthon et al., 2010].

D- and L- lactate were measured in the supernatants after heating to 80 °C for 20min with Biosentec D/L-lactic acid enzymatic kits according to the manufacturer's instructions (Biosentec, Toulouse, France).

Immunochemistry

Tissues sections (4-5 µm) of fixed ileum and proximal colon were double-stained with a goat polyclonal antibody raised against GLP-1 diluted at 1/200 (Santa Cruz Biotechnology, CA, USA) and a rabbit anti-chromograninA (ChrgA, diluted at 1/1000 (Immunostar WI, USA). Anti-goat and anti-rabbit fluorescent secondary antibodies (1/1000) were used to reveal GLP-1 and chromograninA labeling, respectively. Nuclei were counterstained with DAPI. Tissues sections were mounted in Prolong Gold anti-fading medium (Molecular Probes, Thermo Scientific, Courtaboeuf, France). Three sections were analyzed per sample using the Nanozoomer (x20) (Hamamatsu, Japan). Number of fluorescent cells was counted twice by a blind operator, along the crypt-villus axis unit, in 3 different sections, using the NDP view software (Hamamatsu). A total of 40 to 60 crypt-villus units were counted per section.

Quantitative real-time PCR

Total RNA extraction from ileum and colon was performed using "QIAamp RNA Blood Mini" kit (Qiagen, Courtaboeuf, France) according to manufacturer instructions. Two micrograms RNA were reverse-transcribed using M-MLV reverse transcriptase (Promega, Charbonnières-les-Bains, France). Five microliters of 1/40 dilution of cDNA solution were subjected to RT-qPCR in a Bio-Rad iCycler iQ system (Biorad, Marnes-la-Coquette, France) using the qPCR SYBR Green Eurobiogreen®Mix (Eurobio, Les Ulis, France). Quantitative PCR consisted of 40 cycles, 15 s at 95°C, 15 s at 60°C and 15 s at 72°C each. Primers sequences are figured in Table S1 in Supplementary Material. For Neurog3 quantification, PrimePCR™ SYBR® GreenAssay: Neurog3, rat (Biorad) was used. Relative mRNA quantification was expressed using the $2^{-\Delta\Delta cq}$ method with actin gene as a reference.

Bacterial 16S sequencing of cecal contents

DNA was extracted from pellets of ceco-colonic contents (max 250 mg, using the QIAamp Fast DNA Stool Mini kit (Qiagen, Hilden, Germany) after enzymatic and mechanical disruptions as described previously [Fança-Berthon et al 2010] except that homogenization was carried out at 7800 rpm for 3x 20 s intervals with 20 s rest between each interval in a Precellys® "evolution" bead-beater (Bertin, Montigny-le-Bretonneux France). The V4 hyper-variable region of the 16S rDNA gene was amplified from the DNA extracts during the first PCR step using composite primers (5'-CTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTAA-3' and 5'-GGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3') which were based on the primers adapted from Caporaso et al (2011; i.e. 515F and 806R). Amplicons were purified using PP201 PCR Purification Kit (Jena Bioscience, Germany). Paired-end sequencing was performed on a HiSeq 2500 System (Illumina, San Diego, CA, USA) with v3 reagents, producing 250 bp reads per end, according to manufacturer's instructions at the GeT-PlaGe platform (INRA-Toulouse, France). The 16S rDNA raw sequences were analyzed with the FROGS v2 pipeline (http://frogs.toulouse.inra.fr/) [Combes et al 2017; Escudié et al 2018]. After de-multiplexing, quality filtering and chimera removing, the taxonomic assignments were conducted for OTUs with abundance > 0.005% with Blast using Silva 128 database containing sequences with a pintail score at 80 to determine the bacterial compositions.. FROGSSTAT Phyloseq tools were used to normalize raw abundances by rarefaction and to calculate alpha and beta diversity indices.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA) or R (librairies "stats v3.5.1" and "corrplot v0.84", [R Core Team 2018]). Differences among treatments were searched using one way ANOVA followed by Tukey's multiple comparisons tests for most data with the exception of growth and food consumption data that have been subjected to multiple t-tests with correction for multiple comparisons using the Holm-Sidak method. Sweet taste preference test was analyzed by the one sample t-test to compare values to 50% (no preference). A p value < 0.05 was considered statistically significant.

3. Results

3.1. Neonatal OS supplementations did not substantially affect rat growth

Both FOS and αGOS supplementations were associated with a significant transitory reduction of growth of pups in the early days of the intervention (PND7 to PND10 and PND6 to PND8, respectively, Figure S1). When compared with body weights from the CTL group, these observed differences only reached 9.1 to 11.5% and did not significantly affect the cumulative weight gains measured either from birth until the end of the supplementation or for the whole lactation period (Table 2).

Table 2. Bodyweight gain (g) during lactation.

Treatment	BW gain PND0-14	BW gain PND0-20
CTL	30.4 ± 4.2 [1]	50.5 ± 6.0
FOS	28.0 ± 3.4	45.7 ± 4.6
GOS/In	29.4 ± 3.3	49.6 ± 5.8
αGOS	27.9 ± 2.7	46.8 ± 5.1

[1] Data are means ± SD collected from the total effective of rats (n=15-16 per group).
BW, bodyweight.

No significant differences in bodyweight were observed between groups after weaning (Figure S2).

3.2. Neonatal OS supplementations exerted a marked immediate impact on intestinal environment.

3.2.1. OS supplementations modified both composition and activity of neonatal intestinal microbiota

Following 16S sequencing, no significant differences were noticed in raw sequences numbers between cecocolonic samples collected at PND14/15 (355245 ± 10367, 30306 ± 13817, 40275 ± 18343 and 31808 ± 10101 for CTL, FOS, GOS/In and αGOS, respectively) or in percentages of sequences kept after quality filtering (83.8 ± 4.0, 76.4 ± 18.4, 83.9 ± 4.2, and 81.7 ± 7.1). Cecocolonic contents of the animals supplemented with OS exhibited similar reductions in richness ($p < 0.001$) as compared with the CTL animals (Chao1 values: 66.2 ± 21.0, 72.9 ± 28.1, and 73.9 ± 35.3 for FOS, GOS/In and αGOS, respectively *versus* 180.0 ± 35.7 for CTL). The cluster dendrogram generated using weighted UniFrac metric which illustrate beta or between-sample diversity, highlighted an obvious dissimilarity between the microbiotas of the OS-supplemented animals and those of the animals from the CTL group (Figure 1) but did not allow evidencing any effect of the nature of the OS.

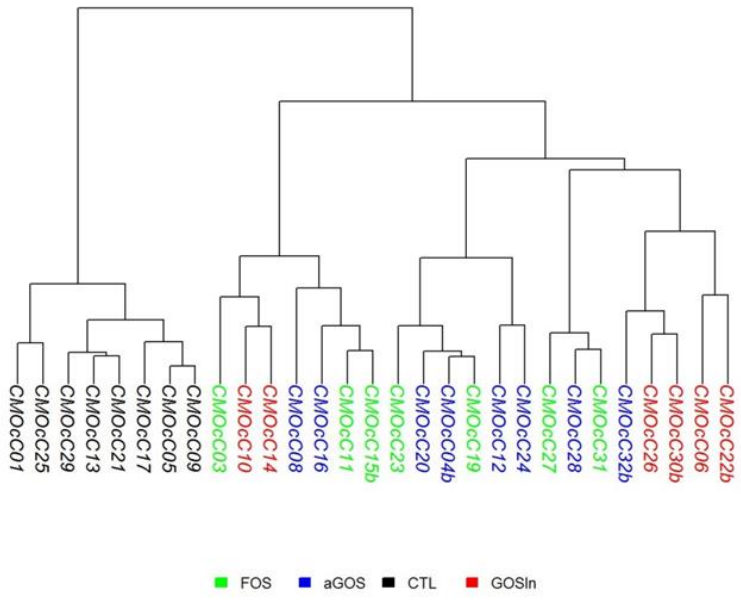

Figure 1. Hierarchical clustering based on the Ward's method of phylogenetically informed distance matrix computed using the weighted UniFrac metric for cecocolonic contents collected at PND 14/15 (n=6 to 8 per group.).

When considering families occurring at more than 0.01% of the total sample abondances (Table 3) OS impact was typified by significant decreases in Lactobacillaceae, Bacteroidales S24-7 group, Prevotellaceae, Streptococcaceae, Peptococcaceae, Coriobacteriaceae, Aerococcaceae, Family XIII, and Rikenellaceae. Moreover, OS supplementation decreased Ruminococcaceae but this impact reached statistical significance for FOS and αGOS only. These decreases in relative abundances were differently compensated according to the OS: increases in Bifidobacteriaceae reached statistical significance following FOS and αGOS supplementations, Enterobacteriaceae were increased following αGOS supplementation and Lachnospiraceae were increased following GOS/In supplementation.
Significant differences within OS were scarce and only occurred between GOS/In and αGOS in their impacts on Lachnospiraceae (Table 3).

Table 3. Relative abundances (%) for families with abundances > 0.01% at PND14/15 according to the postnatal OS supplementation.

Family	CTL	FOS	GOSIn	αGOS
Actinomycetaceae	0.095±0.084 [1]	0.028±0.032	0.076±0.056	0.068±0.058
Aerococcaceae	0.086±0.029 [a]	0.011±0.011 [b]	0.015±0.012 [b]	0.020±0.019 [b]
Alcaligenaceae	0.020±0.041	0.030±0.047	0.378±0.576	0.036±0.053
Bacteroidaceae	2.352±0.991	6.510±10.047	6.837±5.729	3.719±5.492
Bacteroidales.S24.7.group	6.812±2.953 [a]	0.053±0.080 [b]	0.098±0.094 [b]	0.084±0.103 [b]
Bifidobacteriaceae	0.624±0.45 [a]	17.188±12.735 [b]	7.894±7.947 [ab]	13.577±10.631 [b]
Campylobacteraceae	0.009±0.024	0.093±0.220	0.066±0.151	0.294±0.546
Clostridiaceae.1	0.273±0.146	2.413±3.231	5.509±8.749	5.044±4.581
Coriobacteriaceae	0.108±0.039 [a]	0.039±0.034 [b]	0.036±0.041 [b]	0.023±0.018 [b]
Corynebacteriaceae	0.032±0.023	0.007±0.011	0.020±0.030	0.012±0.023
Desulfovibrionaceae	0.098±0.182	0.000±0.000	0.003±0.008	0.006±0.014
Enterobacteriaceae	13.86±5.97 [a]	23.48±12.23 [ab]	19.51±6.69 [b]	33.42±11.99 [b]
Enterococcaceae	0.435±0.707	0.145±0.203	2.892±6.293	0.542±0.771
Erysipelotrichaceae	0.682±0.387	4.080±3.988	3.774±4.950	2.766±3.002
Family.XIII	0.062±0.030 [a]	0.004±0.008 [b]	0.000±0.000 [b]	0.001±0.003 [b]
Lachnospiraceae	6.327±2.300 [a]	9.787±6.180 [ab]	15.298±9.544 [b]	4.962±4.587 [b]
Lactobacillaceae	57.47±8.72 [a]	28.74±10.84 [b]	24.47±5.71 [b]	31.13±11.24 [b]
Micrococcaceae	0.140±0.064	0.075±0.074	0.071±0.053	0.110±0.105
Pasteurellaceae	0.582±0.581	0.236±0.235	0.456±0.297	0.394±0.446
Peptococcaceae	0.396±0.182 [a]	0.006±0.015 [b]	0.015±0.019 [b]	0.007±0.021 [b]
Peptostreptococcaceae	0.747±0.485	0.471±0.262	0.543±0.108	0.640±0.379
Porphyromonadaceae	1.242±1.153	5.924±9.747	9.826±15.228	2.055±5.475
Prevotellaceae	2.136±1.540 [a]	0.014±0.016 [b]	0.011±0.018 [b]	0.028±0.060 [b]
Rikenellaceae	0.034±0.039 [a]	0.001±0.004 [b]	0.000±0.000 [b]	0.001±0.003 [b]
Ruminococcaceae	3.242±0.743 [a]	0.135±0.147 [b]	1.610±2.622 [b]	0.406±0.665 [b]
Streptococcaceae	2.118±0.620 [a]	0.510±0.316 [b]	0.586±0.156 [b]	0.643±0.450 [b]

[1] Data are means ± SD (n=6 to 8 per group). Within a row, values followed by different letters differ significantly ($p < 0.05$).

In agreement with these compositional changes, the 10 days supplementation deeply affected fermentation end-products concentrations in both ileal and colonic contents at PND14/15.

In the ileum, lactate concentration was below the detection limit (0.22mM) in all animals and the concentration of acetate, the sole SCFA present at this age in this intestinal segment, was significantly increased ($p < 0.005$) by FOS supplementation (6.9 ± 3.6 mM) as compared with CTL (0.3 ± 0.4 mM), GOS/In (1.6 ± 2.0 mM) and αGOS (0.6 ± 0.8 mM).

In the cecum, the concentration of total end-products was increased in all OS groups as compared with CTL (Figure 2) This was mainly due to an increase in SCFA concentration, which reached statistical significance only with FOS and also, for αGOS only, to an increase in lactate concentration.

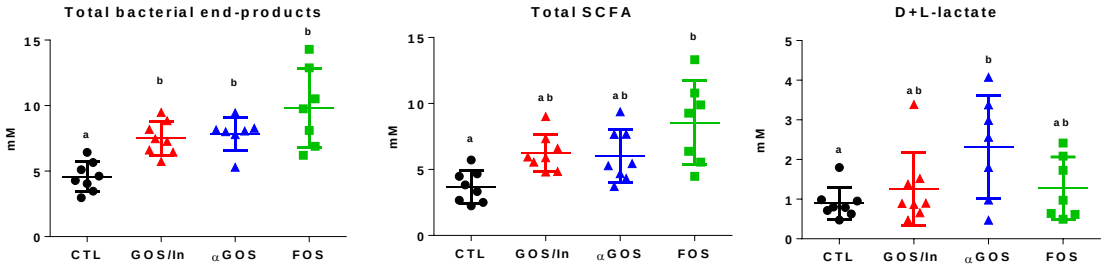

Figure 2. Cecocolonic concentrations of fermentation end-products. Individual, mean and SD values are plotted (n= 7 to 8 per group). Different letters indicated significant difference ($p < 0.05$) between groups.

Increases in total SCFA reflected acetate ones which were significantly increased by both FOS and GOS/In and were paralleled by significant decreases of pH values (Table 3). Moreover, OS supplementation shifted microbiotal activity as illustrated by significant changes in the relative proportions of acetate (93. 8± 4.6, 93.1 ± 4.1, and 95.4 ± 2.9 % for FOS, GOS/In and αGOS, respectively *versus* 86.3 ± 4.5 % for CTL) and propionate (5.4 ± 4.6, 5.2 ± 3.4, and 3.6 ± 2.9 % for FOS, GOS/In and αGOS, respectively *versus* 10.7 ± 3.0 % for CTL). Concentration and relative proportion of butyrate - which is scarcely produced in neonatal stage - were not significantly affected by supplementations.

Table 4. Concentration (mM) of major SCFA in cecocolonic contents at PND 14/15.

Treatment	Acetate	Propionate	Butyrate	pH
CTL	3.17± 1.05^{a}	0.39± 0.16	0.07± 0.04	6.9±0.3^{a}
GOS/In	5.82± 1.32^{b}	0.33± 0.21	0.10± 0.09	6.3±0.2^{b}
αGOS	5.69± 1.77^{ab}	0.28± 0.27	0.05± 0.00	6.1±0.2^{b}
FOS	8.00± 2.94^{b}	0.47± 0.37	0.06± 0.04	6.2±0.2^{b}

[1] Data are means ± SD (n=7 to 8 per group). Within columns, values followed by different letters differ significantly at $p < 0.05$.

3.2.2. OS supplementations modified both differentiation and activity of the neonatal EEC

In the ileum, a profound effect on the enteroendocrine lineage was induced by the neonatal OS supplementations as revealed by a significant decrease in *Neurog3* expression in the OS groups when compared with CTL, whereas early expressed marker in the commitment secretory lineage (*Atoh1*) was not significantly affected (Figure 3). Relative expression of genes specifically implied in the differentiation of EEC (*Pax4* and *Pax6*) was significantly decreased in OS supplemented groups as compared with CTL whereas expression of *Foxa1* did not vary between groups. Similarly to *Pax4* and *Pax6*, *Neurod1* expression was reduced in OS groups as compared with CTL but this did not reach statistical significance for FOS. With regard to expression of genes coding for peptides produced by mature L-cells, *Pyy* was significantly increased in OS groups as compared with CTL. Meanwhile, despite a 2 fold increase in *Gcg* expression in OS groups compared with CTL, this effect was not statistically significant due to a great variability of expression between samples.

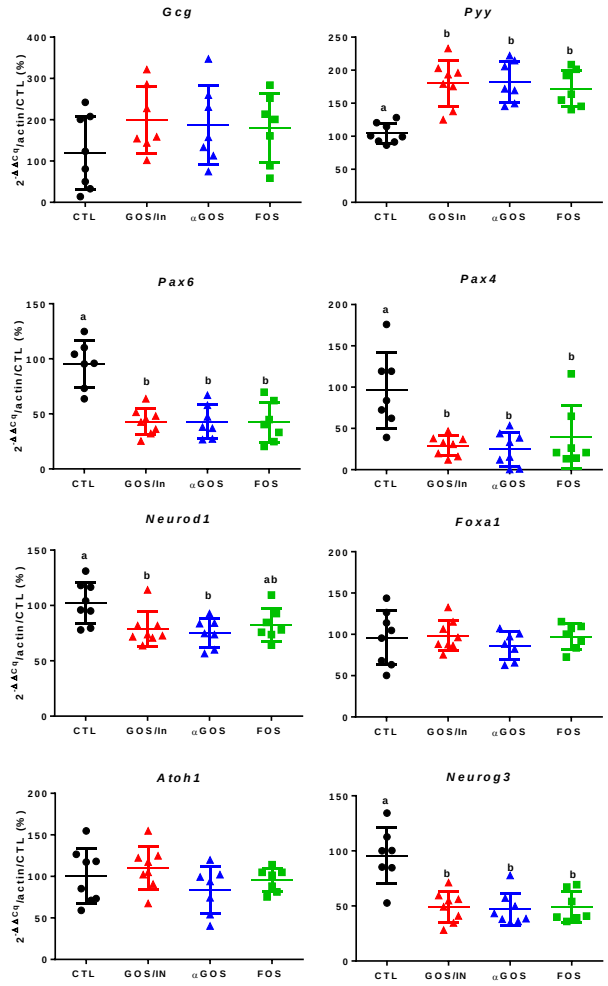

Figure 3. Relative expression of gene implied in the endocrine lineage and in L-cells differentiation in the ileum. Different letters indicate significant difference between groups ($p < 0.05$). Data are fold-change expressed in % of CTL group. Individual, mean and SD values are plotted (n= 7 to 8 per group).

In the proximal colon, the impact of OS supplementations was much more moderated and their only significant effect was a decrease in the expression of *Pax4* (Figure S3).
Concomitantly with this profound remodeling in the expression of markers of L-cells differentiation, the number of GLP-1/ChgrA positive cells, i.e. mature EEC, was higher in the ileum of pups from OS groups as compared with CTL but this reached statistical significance for villi only (Figure 4).

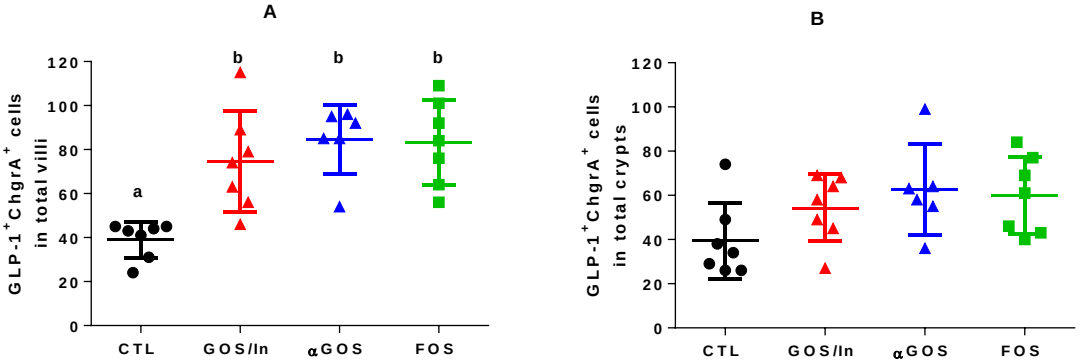

Figure 4. Effect of OS supplementation on the density of GLP-1 cells in ileum: (A) in villi (**B**) in crypts. Different letters indicate significant differences among groups ($p < 0.05$); Individual, mean and SD values are plotted (n= 6 to 7 per group).

In agreement with this rise in the number of mature EEC, plasma concentrations of GLP-1 (Figure 5A) and PYY (Figure 5B) were significantly increased by all the neonatal OS supplementations, as compared with CTL

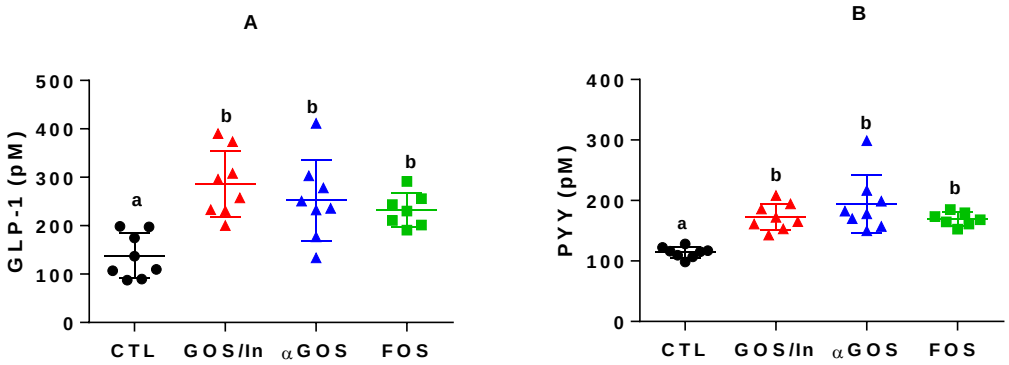

Figure 5. Plasma concentration of (A) Total GLP-1; (B) Total PYY at PND 14/15. Different letters indicate significant differences among groups ($p < 0.05$). Individual, mean and SD values are plotted (n= 7 to 8 per group).

Significant positive associations between plasma concentrations of GLP-1 and PYY and the ileal expressions of their respective genes were evidenced (Figure 6A). Conversely, these plasma concentrations as well as the densities of GLP-1 secreting cells were inversely correlated with expressions of *Neurog3*, *Neurod1*, *Pax4*, and *Pax6*. With respect to associations between microbiota and EEC descriptors (Figure 6B), only some of the differentiating factors (*Pax4, Neurod1, Pax6* and *Neurog3*) exhibited significant positive correlations with the abundances of some bacterial families that correspond to those whose abundance was significantly reduced by OS, except for Prevotellaceae. For these factors, the sole negative correlation was that between *Neurod1* and abundance of Clostridiaceae.1. Conversely, PYY and GLP-1 plasmatic concentrations, EEC densities and *Pyy* expression, but not *Gcg* expression, were negatively correlated with the same families including Prevotellaceae.

Altogether, these results indicate that OS supplementations profoundly modulate the neonatal microbiota, both in its composition and fermentative activity with repercussions not only in the cecocolon but also, as exemplified with FOS, in the ileum. Increased density of ileal L-EEC and their secreted anorectic hormones, GLP-1 and PYY were observed and concomitantly but unexpectedly, expression of transcription factors beyond the stage of secretory cell engagement (Atoh1) was inhibited. Whether this strong impact of early OS supplementation on satiety peptides-related EEC could last later in life and affect eating behavior has been further investigated.

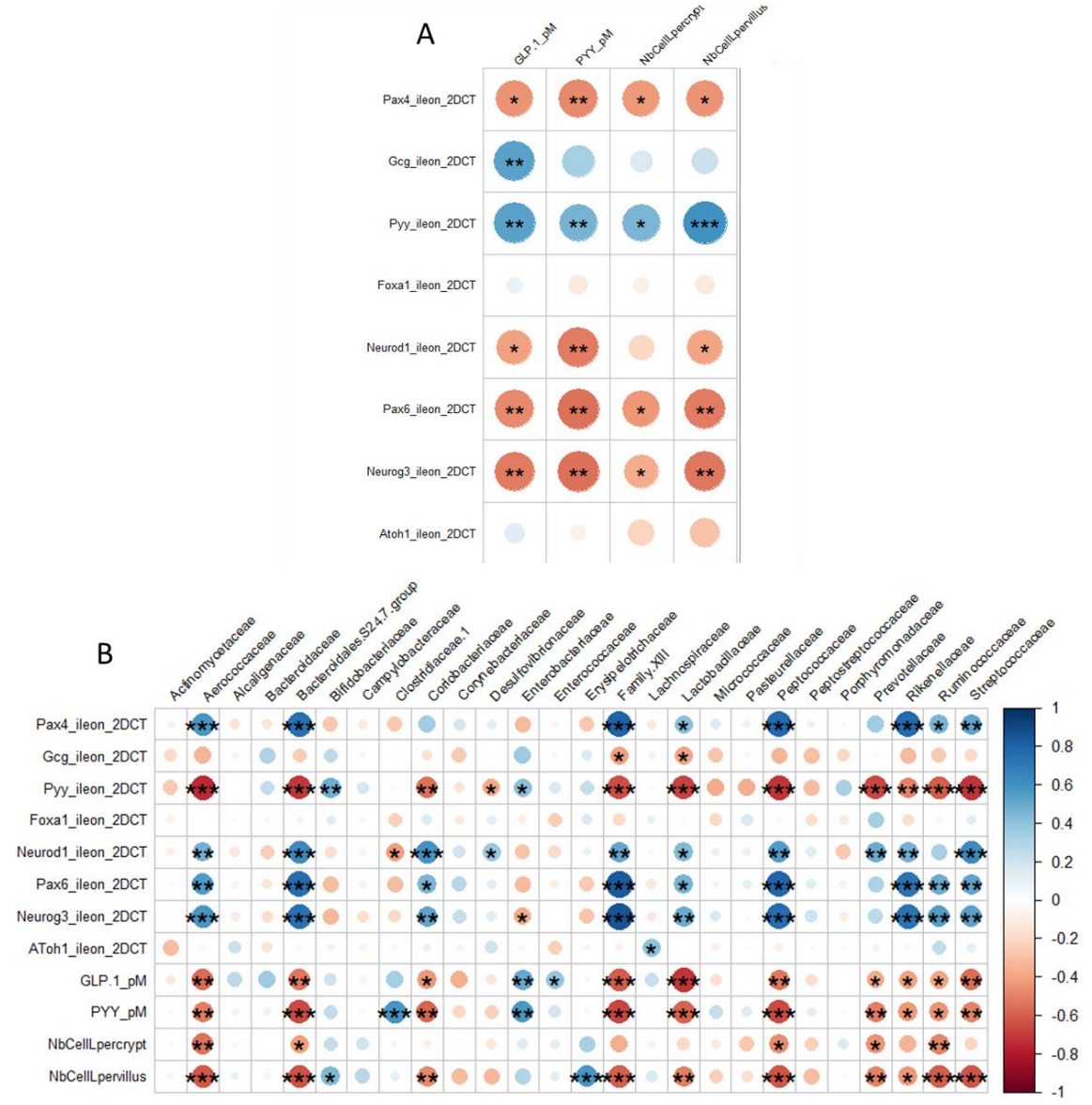

Figure 6. Correlograms within EEC descriptors (A) or between these descriptors and the relative abundances of main bacterial families (B). Positive correlations are displayed in blue and negative correlations in red. The intensity of the color and the size of the circles are proportional to the correlation coefficients. Asterisks indicate the level of significance (*, p< 0.05; **, p < 0.01; ***, p< 0.001). On the right of the correlogram, the color legend shows the correspondence between correlation coefficients and colors.

3.3. Neonatal OS supplementations had no significant long-term consequences

3.3.1. Neonatal OS supplementations did not significantly program enteropeptides production or eating behavior in adulthood

To investigate the long-term effect of a neonatal supplementation of OS on nutrient sensing in EEC, once pups reached adulthood, we studied the release of GLP-1 and PYY in response to both a 20 min- test meal (PND 74/76) and an oral bolus of glucose (PND 124/126) after a 16h-fasting.

No significant differences were observed between groups in the amount of food consumed during the 20min test meal (data not shown). In response to this meal, plasma concentration of GLP-1 increased immediately after refeeding and returned to pre-prandial level between 120 and 180 minutes later (Figure 7A). Total amount of secreted GLP_1 during that period, quantified by AUC, did not significantly differ between groups (Figure 7B). PYY secretion did not show any postprandial peak or significant differences between groups (data not shown).

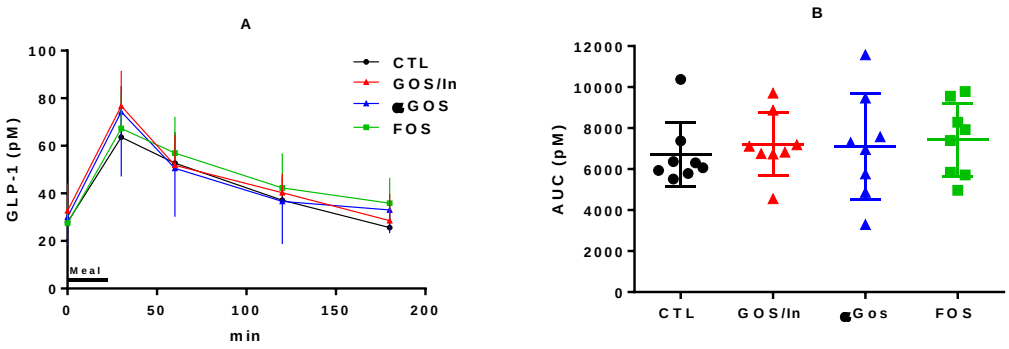

Figure 7. GLP-1 secretion in response to a 20 min-meal. (A) Plasma concentration of total GLP-1 measured during the 3h-kinetic follow-up (means ± SD); (B) Total amount of GLP-1 secreted during the 0-180min period expressed as AUC. Individuals, means and SD are plotted. (n=7 to 8 per groups in A and B).

Similarly, at PND 124/126, plasma concentrations of GLP-1 (CTL: 34.4 ± 13.5; GOS/In: 38.6 ±28.6; αGOS: 28.9 ± 10.0 and FOS: 37.9 ± 20.6 pM) and PYY (CTL: 84.7 ± 4.0; GOS/In: 88.7 ± 7.8; αGOS: 91.4 ± 7.3 and FOS: 91.5 ± 5.8 pM) measured 2h after an oral bolus of glucose did not show any significant difference between groups.

To investigate the long-term effect of a neonatal supplementation of OS on later eating behavior, we followed up the food consumption from weaning to adulthood, performed a refined analyze of feeding pattern using physiological cages from PND75 to PND100 and assessed preference for the sugar taste between PND109 and PND111.
The analysis of food consumption during development only revealed a single significant difference which occurred at PND32 between animals from the FOS and the CTL groups (Figure 8), what indicate that neonatal supplementation with OS did not significantly program the subsequent food intake in our experimental conditions.

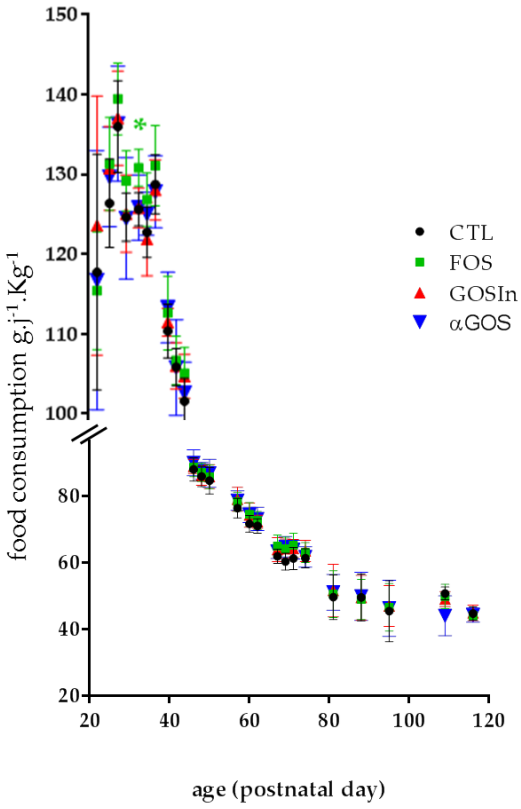

Figure 8. Daily consumption of food during post-weaning. The asterisk indicates a significant difference between FOS and CTL groups ($p < 0.05$). Data are means ± SD (n=7 to 8 by group and day).

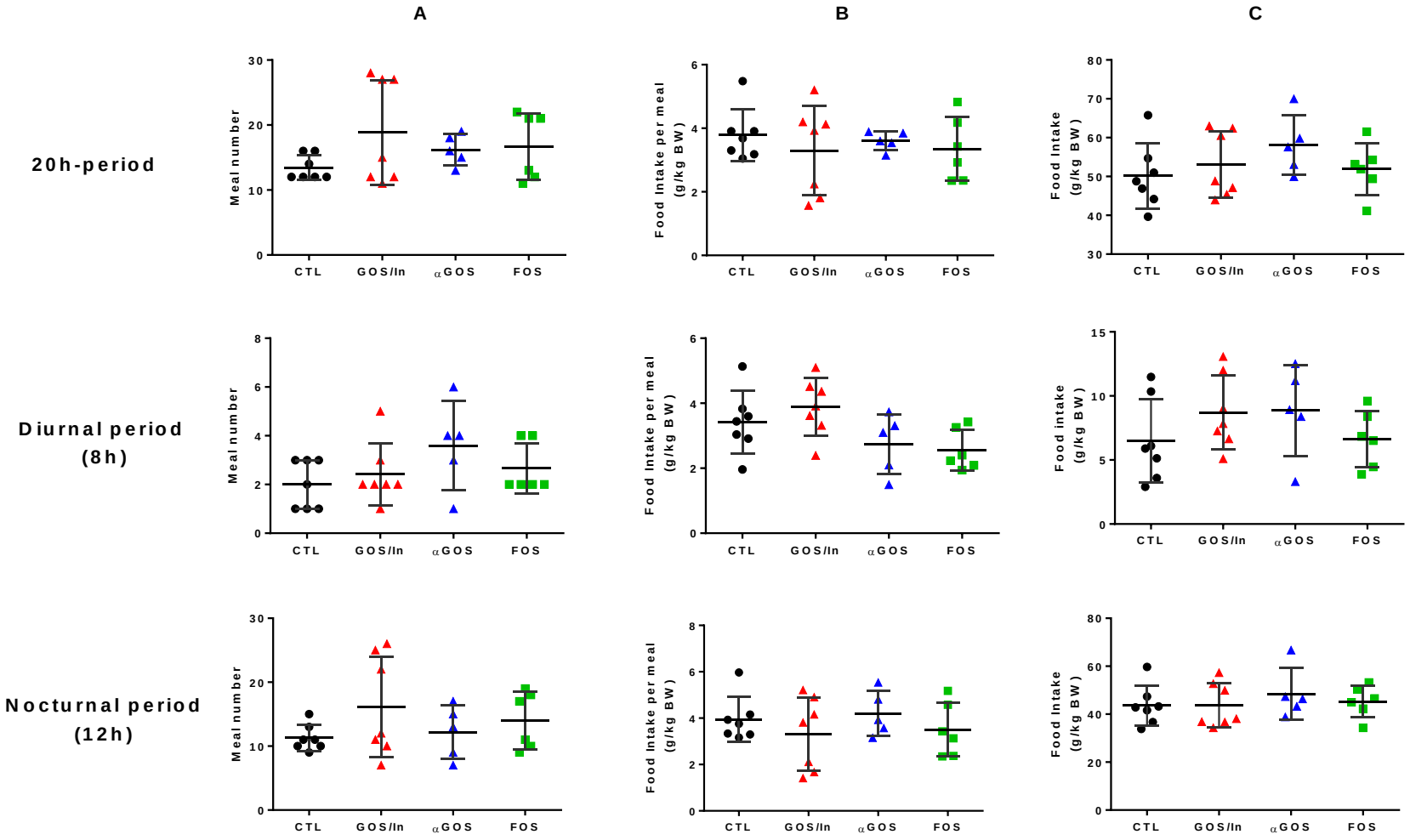

Figure 9. Feeding patterns illustrated by (**A**) Meal number; (**B**) Food intake per meal; (**C**) Food intake during the considered period analyzed in physiological cages at PND 75-100 (n=5-7 per group). BW, bodyweight. Individuals, means and SD are plotted (n=5 to 8 per groups).

This lack of effect on daily food consumption was confirmed by the detailed analysis of food consumption: we observed no significant difference in meal patterns among groups, whatever the period of measurement (total 20h period of measurement, diurnal period (8h) or nocturnal period (12h) (Figure 9).

During sweet taste preference test, there was no significant difference between groups with regard to the consumption of saccharin solution expressed as percentage of daily drink whatever the test day considered (Figure 10). Strikingly, the preference for sweet test for the GOS/In group did not reach statistical significance on the first test day, in contrast to FOS and αGOS groups. However, this preference did not persist on day 2, conversely to what was observed for the CTL group. This thus suggests that neonatal supplementation of OS slightly reduced the duration for sweet preference at adulthood.

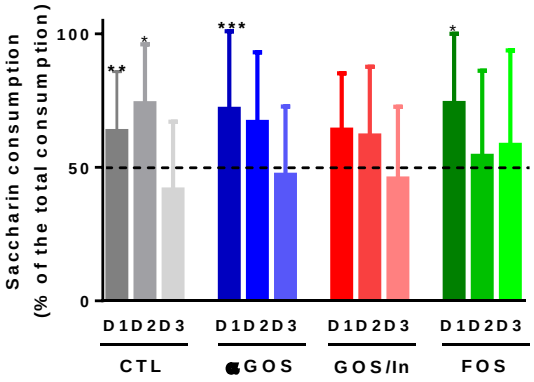

Figure 10. Preference for sweet taste. Data are means ± SD (n=7 to 8). Asterisks represent significant preference as compared with no preference (i.e. 50%, dotted line): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.3.2. Neonatal OS supplementations did not significantly program adult intestinal microbiota

At adult age (PND 124/126), no significant differences were observed between treatments with respect to raw number of sequences obtained, percentages of sequences kept after quality filtering, or alpha-diversity indexes (data not shown). Similarly, β-diversity analysis, principal component analysis on OTUs abundances (Figure S4) as well as comparisons of the cumulated relative abundances at the family level (Figure 11) failed to show any significant difference between cecocolonic samples according to the neonatal supplementation. Finally, neither ileal nor cecocolonic concentrations of SCFA showed significant differences between experimental groups (Table S2).

Altogether, these data did not support any programming impact of neonatal OS supplementation on adult microbiota.

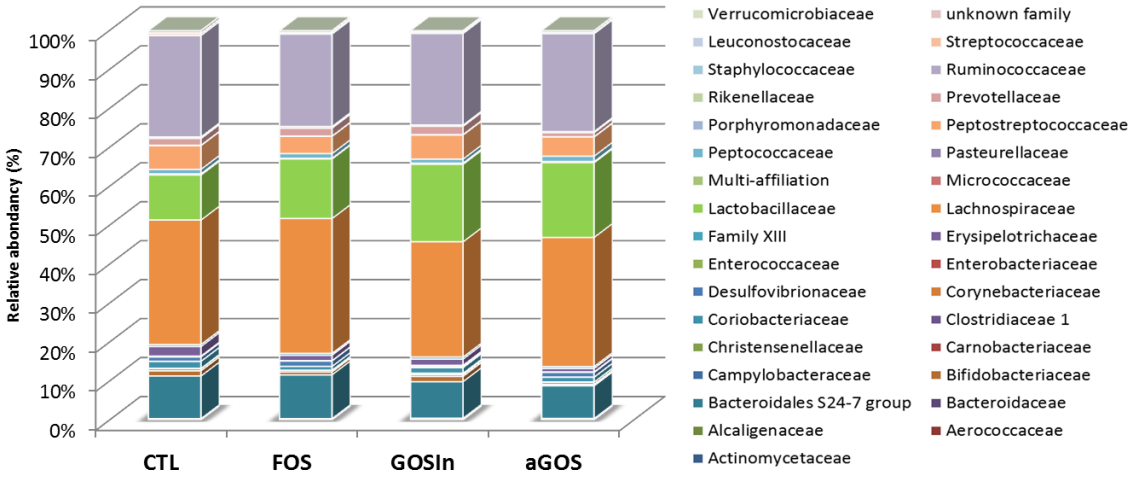

Figure 11. Impact of postnatal OS-supplementation on cecocolonic microbiota composition at PND124/126: families distribution expressed as averaged cumulated relative abundances (n = 7 to 8 per group).

4. Discussion

Considering that regulation of feeding behavior, could possibly be programmed by early life and controlled by intestinal microbiota, we hypothesized that neonatal microbiota modifications may program adult feeding behavior. We thus assessed the ability of nutritionally-induced intestinal microbiota modulations to affect the maturation and functioning of L-EEC in suckling rats and to result in delayed alterations in eating behavior and in GI peptides secretion at adulthood. In this study, we show that neonatal supplementation with 3 different OS strongly impacts cecocolonic microbiota, GLP-1 cells density in the ileum, and production of satiety related peptides in neonatal period, but fails to induce any significant enduring effects at adulthood neither on eating behavior nor gut peptides.

Neonatal OS supplements affected intestinal microbiota despite its immaturity

Corroborating our previous findings based on a non-exhaustive analysis of the microbiota [Morel et al 2015a], and in agreement with several *in vivo* and *in vitro* studies investigating, at adulthood, in humans and animals [see for review Rastall 2010 or e.g.: Liu et al 2017; Wang et al 2017] or in human infants [see for review Skórka et al 2018], the impact of OS (including that of the αGOS [Fehlbaum et al 2018] on intestinal microbiota, all the oligosaccharides used here dramatically affected neonatal microbiota in rat pups. This confirms that the prebiotic property demonstrated in adult rats operates in neonatal pups as well despite the immaturity of the microbiota at this stage of development [Fança-Berthon et al 2010].

Beyond these changes in composition and a reduction in microbiota richness, our neonatal OS supplementations have also modified the activity of the microbiota by stimulating the production of acetate and lactate at the expense of that of propionate. This result stands out from what is observed in adult rats where GOS and FOS are frequently reported as stimulatory for propionate and/or butyrate production [e.g.: Le Blay et al 1999; Overduin et al, 2013]. Such a contradiction probably results from the progressive maturation of the microbiotal capacity to synthesis the different SCFA [Midtvedt & Midtvedt, 1992]. Thus, the production of butyrate is practically undetectable before the 16th day of life in rat [Fança-Berthon et al 2010]. Anyway, the neonatal OS supplementations we performed resulted in microbiotas that markedly differ from that of unsupplemented animals, what was a prerequisite for investigating the ability of neonatal microbiota modulation to program adult eating behavior or gut peptides response.

OS supplements would stimulate ileal EEC to produce GLP-1/PYY while acting in feedback on endocrine precursors

Our results showed that neonatal OS supplementation had immediate effects on ileal mature GLP-1-cells by increasing their density in villi and the mRNA expression of Gcg and PYY leading to enhanced plasma concentrations of these two anorectic peptides. These new observations in neonatal rats are consistent with those reported in adult rats for FOS and GOS/In [Cani et al 2004; Cani et al, 2007; Parnell & Reimer 2012; Delzenne et al 2007, Overduin et al 2013] and are, to our knowledge, reported here for the first time for αGOS. In one of these previous studies, this increased production of GLP-1 was related to higher differentiation of *Neurog3*-expressing EEC progenitor into L-cells in colon [Cani et al, 2007]. Here, we demonstrated a drastic downregulation of endocrine lineage-devoted genes during OS supplementation, mainly in the ileum. This unexpected result is somewhat complicated to conciliate with an effect of OS on endocrine early precursor leading to produce more L-cell subtype.

Neurog3 marks the endocrine progenitor and is essential for generating newly formed EEC [Li et al., 2011]. Post-neurog3 differentiation and maturation of EEC is controlled by dynamics in transcriptional factors such Neurod1, Pax4 and Pax6 and many others (Arx, Pdx1, Foxa1 and Foxa2). The hierarchy of these events is still poorly understood [Schnonhoff et al, 2004; Beucher et al, 2012, Engelstoft et al, 2013] and the extrinsic factors that may interplay remain largely unknown. In the present study, the well-known effect of OS prebiotics to stimulate L-cells cannot be simply related to an impact on endocrine precursors as suggested in the previously mentioned study [Cani et al, 2007]. As we know that *Neurog3* expression is restricted to immature proliferative cells, the decreased *Neurog3* expression we observed in ileum may rather reflect a feedback regulation to limit new EEC generation in response to OS supplementation. A similar observation (decrease in duodenal Neurog3 and increased density of EEC) was reported in a model of maternal deprivation [Estienne et al., 2010]. These data and ours suggest that the postnatal environment impact differentiating EEC precursors and not proliferative progenitor, to increase EEC density. High level of circulating GLP-1 has been previously related to increased number of ileal L-cells in Gcgr-deleted mice and this effect involved up-regulation of post-neurog3 transcription factors, affecting proliferation of L-cells precursors [Grigoryan et al., 2012]. Here, expression of these factors namely Neurod1, Pax4 and Pax6 was reduced in OS-supplemented groups with high circulating levels of GLP-1, suggesting a different mechanism in the increased density of L-cells. In this respect, it should be noted that although EECs are still classified according to their major/unique hormone product (as for example GLP-1 for L-cells), it is now acknowledged that EEC are multihormonal [Habib et al., 2012; Engelstoft et al., 2013; Grunddal et al., 2016]. In particular, very recent data demonstrated that mature differentiated EECs display hormonal plasticity allowing them to change their hormonal products in response to extrinsic factor, such as bone morphogenic proteins (BMP), during their migration along the crypt-villus axis [Beumer et al., 2018; Gehart et al., 2019]. Thus, the increased L-cell density observed here may be the result of direct effect of OS on this plasticity to produce more GLP-1, independently of early markers of EEC proliferation and differentiation. Interestingly, in the present study, the production of CCK, a key early-satiety peptide, was not affected by OS supplementation (data not shown) reinforcing the specific impact of OS on EEC to produce GLP-1 and PYY in a segment of gut

where CCK is not predominantly produced. How OS can modulate both EEC subtypes identity and/or GI peptide expression by acting on extrinsic factor (such villus-produced BMP) need to be further investigated.

What are the putative mediators of the massive effect of neonatal OS supplementation on ileal L-cells?

The identification of the small intestine, rather than the colon, as a privileged site for the action of the OS on transcriptional activity that we observed, has been previously reported in studies involving adult animals [Everard et al. 2011; Cani et al 2009a]. Conventionalization of germ-free mice led to similar observations [Larsson et al., 2012; Sommer et al., 2015; Arora et al., 2017]. However, a nutritional modulation as in our study by OS supplementation may differently impact ileal epithelium as compared to absence/presence of microbiota. As for example, in the Arora's study, conventionalization of germ-free mice led to downregulation of GLP-1 secreting vesicle process in L-cells whereas we observed an increase in GLP-1 and PYY production. These opposite data may also result from great differences in age of animals in these studies. Nevertheless, our data raise the question of how OS modulation of microbiota could act on ileal L-cells. The well- known capacity of SCFA (mainly butyrate but also propionate or even, non-consensually, acetate) to stimulate PYY and/or GLP-1 production [Plaisancié et al 1995; Plaisancié et al 1996; Zhou et al 2006; Zhou et al 2008; Tolhurst et al, 2012; Larraufie et al 2018] seems inconsistent with our observation of a OS-impact mainly localized in the ileum, in a context of no propionate/butyrate synthesis.
Among others potential mechanisms are the acidification of the luminal milieu or changes in pathogens associated molecular patterns (PAMPs). Indeed, Zhou et al (2008) have shown that changes in pH from 7.5 to 6.5 induce *per se* an increase in *Gcg* expression by STC-1 cells *in vitro*. Otherwise, it is known that EECs have receptors for PAMPs (i.e. Toll-like receptors) [see for review van de Wouw et al. 2017 and Plovier & Cani, 2017]. This is of particular interest since Panwar et al [2016] have recently demonstrated that some bacterial strains elicit GLP-1 secretion through signaling agents of the toll-like receptor system, as illustrated by the fact that blockade of MyD88 triggers bacteria-induced GLP-1 secretion.

OS impact on eating behavior, usually observed simultaneously with their consumption, does not seem to be programmable

Despite a certain disparity in the literature [e.g. Hess et al 2011], possibly related to the heterogeneity in dosages or methodologies, several studies have reported beneficial effect of OS prebiotics - mainly fructans but also αGOS, on adult eating behavior, such as attenuated hunger sensation, increased satiety or reduced energy intake in either healthy volunteers [Cani et al., 2009b; Pedersen et al 2013] or overweight adults [Morel et al 2015b; Reimer et al 2017]. To note, the existing literature does not establish whether this is also true in infants who are however frequently supplemented with prebiotics. In agreement with human data, decreased food/energy intakes have been evidenced in adult rodents supplemented with fructans [Cani et al 2004; Parnell & Reimer 2012] or αGOS [Overduin et al 2013]. In both models, these effects have been related to SCFA production by colonic bacteria during OS supplementation. Each of the 3 main SCFA namely, acetate, propionate and butyrate, has been demonstrated to reduce energy intake, particularly in rodent model of diet-induced obesity [Lin et al, 2012; Frost et al 2014, Li et al, 2018] although conflicting results are reported [Perry et al., 2016; Canfora & Blaak 2017], probably depending on the mode (orogastric, intraperitoneal, intracerebroventricular, colonic delivery via fermentable fibers, etc.) and duration (acute *vs* chronic) of SCFA or SCFA precursors administration. In humans, this assumption has been substantiated for both acetate and propionate by numerous studies dealing with appetite-related parameters [see for review Darzi et al 2011] as well as by the observations of reduced hedonic response to high-energy foods regulated in striatum [Byrne et al., 2016] or reduced energy intake following administration of propionate precursors in overweight adults [Chambers et al., 2015; Byrne et al, 2019]. Whether these SCFA regulate appetite directly at hypothalamic level [Frost et al. 2014] or via a vagally-dependent mechanism [Perry et al., 2016; Li et al, 2018] implying or not enhanced intestinal satiety peptide (GLP-1 and PYY) following interaction of SCFA with FFAR receptors on L-cells is still a matter of experimental research in animal models or clinical trials in humans [van de Wouvet al, 2017].
Since each of the actors of the microbiota-EEC-brain axis appears to be long-lastingly impacted by perinatal environment [Estienne et al., 2010; Morel et al, .2015a, Ross and Desai, 2014; Ndjim et al., 2017], we had assumed that early microbiota modulation associated with changes in EEC could program adult eating behavior, what has remained unexplored until now. However, this hypothesis could not be supported in the present study, since eating behavior at adulthood did not appear significantly affected by early life OS supplementation which however had increased total SCFA, concomitantly with increased GLP-1 and PYY release and L-cells density at the end of supplementation. This lack of eating behavior programming indicates that none of the presupposed events (i.e. programming of EEC or vagal sensitivity and/or microbiota programming) has occurred in our conditions. Indeed we did not observe any differences in *c-Fos* expression in the rat brainstem at adulthood 2h after a glucose bolus (data not shown). It thus seems that depending of the nature of perinatal stress (maternal protein restriction [Ndjim et al., 2017] or maternal deprivation [Etienne et al., 2010] or postnatal modulation of microbiota by OS) the long-lasting impact is not systematic. For microbiota, the lack of programming could be related to an inadequacy of the timing when modulation was applied, as discussed below.

Is programming of the microbiota subjected to a particular timing?

In this study, we did not observe any programming impact of any of the neonatal OS supplementations on adult microbiota. This result is in line with what we had previously observed for FOS [Morel et al 2015a] but contradicts the small-scale programming found after neonatal supplementation with GOS/In in this same study. This discrepancy may result from the difference in the method used for analyzing microbiotal

composition, even if it is counterintuitive since 16S sequencing used here is more exhaustive than qPCR used previously. Because this impact was minor, it could also have not been reproduced in our new experimental conditions such as a new batch of animals, a different room of our animal facility, or even possible slight difference in the composition of the semi-purified diets we used, knowing that all these parameters are known to affect the microbiota of laboratory animals [see for review Tomas et al, 2012].
Such a disappearance of the effect, yet drastic, found in our animals at the end of supplementation raises the question of the most favorable period for a sustainable modulation of the composition of the microbiota. Indeed, in our experimental protocols, prebiotic supplementation was applied during a short postnatal period and completed before the start of solid food consumption, whereas the studies reporting programming effects of early supplementation with OS on the subsequent composition of the microbiota were based on longer-term supplements extending from the prenatal period (i.e. supplementation of gestating mothers) to complete weaning or even further [Fugiwara et al 2010; Le Bourgot et al 2019]. Whether the supplementation we applied was insufficiently early, insufficiently late or insufficiently sustained is difficult to establish on the sole basis of this comparison. However, in Fugiwara et al's study, a difference in adult microbiota composition was solely observed for mice offsprings whose supplementation with FOS was maintained beyond weaning. Whether this was also true in Le Bourgot et al's study cannot be evaluated since all piglets were supplemented with FOS for a few weeks after weaning.
From this, one could assume that to be lastingly effective, prebiotics must be able to exert their microbiotal effect after full weaning and thereby control the impacts of new bacterial sources and changes in dynamics of bacterial populations that are resulting from the switch from maternal milk to solid food, which is actually associated with dramatic changes in microbiota composition and activity both in human [Koenig et al 2011] and rat [Inoue & Ushida, 2003; Fança-Berthon et al 2010]. This hypothesis would explain why the early-life events that are known to affect neonatal microbiota composition (i.e. birth mode, infant feeding…) are not associated with significant variations in adult microbiota composition [Falony et al 2016], but it requires strict comparisons between time windows of supplementation to be validated.

All OS studied performed similarly despite differences in their chemical characteristics

In our study, the 3 studied OS have led to very comparable results both in terms of microbiotal impact and physiological repercussions. With regard to microbiotal changes, the observed modifications, in particular the acidification of the contents, the less diversified production of SCFA and the lesser richness of the microbiota, suggest that OS delayed bacterial diversification. This is reminiscent of what supposedly occurs in breast-fed babies compared with babies fed unsupplemented formulas [Mackie et al 1999; Le Huërou-Luron et al 2010]. This similarity is quite surprising in that the chemical nature of the constituent monomers and the pattern of glycosidic linkages in different OS products are expected to influences the abilities of individual bacteria to grow on them [see for reviews Macfarlane et al 2008, Louis et al 2016]. However, our results are consistent with Harris et al's findings [2017], that the orientation of glycosidic linkage is not a main driver of SCFA production profile, that, when it intervenes, its action would rather concern proportion of butyrate, a SCFA poorly produced in our immature animals. Moreover, they also agree with the similarities of microbiotal impacts reported between βGOS and FOS on the one hand [Burokas et al 2017], and between αGOS and βGOS on the other hand [FehIbaum et al 2017].
Thus, our study confirms the prebiotic character of αGOS and, in addition, extends the well-known activity of FOS and GOS/In as satiety enteropeptides secretagogue to this new prebiotic, what accords with its satietogenic effect described in humans [Morel et al 2015b].

5. Conclusions

In conclusion, our study depicts that the ability of OS to modulate the EEC, previously described in adults, also operates in the neonatal period, despite the immaturity of the microbiota at this time. This observation thus calls into question the nature of the mediators actually involved, as supposed until now. In addition, our in-depth study of the OS-impacts on the genes regulating the differentiation of EEC precursors questions the current understanding of the ontogenesis of these cells.
Finally, our results do not demonstrate any programming effect of OS neither on EEC and food consumption nor on adult microbiota constitution. If this holds true for humans, this is reassuring considering that the current study investigated type and doses of OS mimicking some of those commonly prescribed in formula for toddlers.

Supplementary Materials:
Figure S1: Postnatal growth of suckling rats in the different groups of OS supplementation,
Table S1: Primers sequences,
Figure S2: Growth of weaned rats until adulthood,
Figure S3: Relative expression of gene implied in the endocrine lineage and in L-cells differentiation in the colon.
Figure S4: Hierarchical clustering based on the Ward's method of phylogenetically informed distance matrix computed using the unweighted UniFrac metric for cecocolonic contents collected at PND 124/126,
Table S2: Concentration of SCFA in ileal and ceco-colonic contents at PND 124/126

Author Contributions: C.M., G.L.D and M.R.D conceived and designed the experiments with the help of PP; C.M., M.R.D., GLD and A.L.P. performed the experiments; A.L.P., A.P. and I.G. contributed to biological analyses; C.M., G.L.D and H.B. analyzed the data and prepared figures; G.L.D. and C.M. drafted the manuscript. E.C. and P.P. revised the manuscript.

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Conflicts of Interest: The authors declare that they had a financial relationship with the organization that partly sponsored the research. E.C. is employee of Olygose.

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Supplementary data

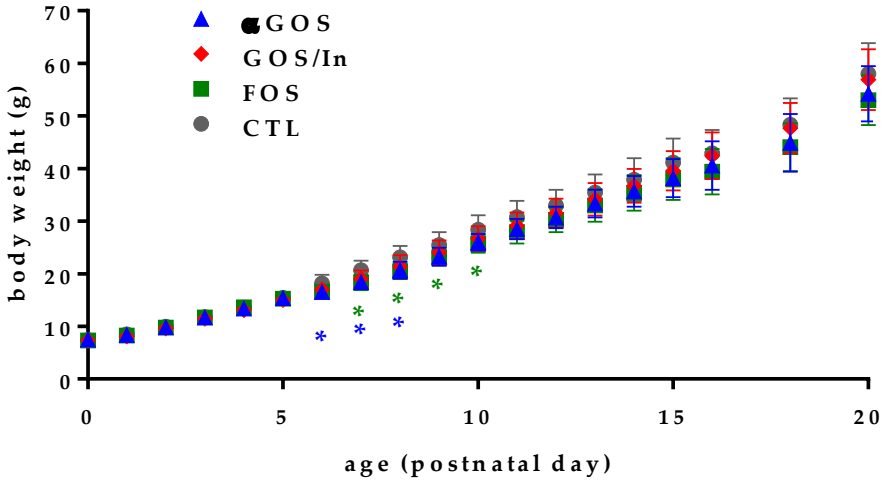

Figure S1. Postnatal growth of suckling rats in the different groups of OS supplementation. Data are means +/- SD collected from the total effective of rats (n=15-16 per group). Asterisks indicate significant difference compared with CTL, their color refer to the treatment received between PND 5-14/15.

Target	Forward primer	Reverse primer	Accession Number
β-actin	CTATCGGCAATGAGCGGTTCC	GCACTGTGTTGGCATAGAGGTC	NM_031144
Atoh1	TGTTAGCAACG TGTGACTTC	CAGACCAGAGACAGAGATACG	NM_001109238
Gcg	CTAATGCTGGTACAAGGCAG	GTGAATGTGCCCTGTGAATG	NM_012707
Neurod1	CACGCAGAAGGCAAG	TGGTCATGTTTCCACTTCCTGT	NM_019218
Foxa1	GTTCCGCACAGGGTTGGATA	CTGACCGGGACAGAGGAGTA	NM_012742
Neurog3	Not available	Not available	NM_021700
Pax4	CCCAAGGGTATTGGGGGAAG	GGATACACTGGGAGCCTTGTC	NM_031799
Pax6	ATACCTACACCCCTCCGCAC	TGAGTCCTGTTGAAGTGGTTCC	NM_013001
PYY	AGCGGTATGGGAAAAGAGAAGTC	ACCACTGGTCCACACCTTCTG	NM_001034080

Table S1. Primer sequences

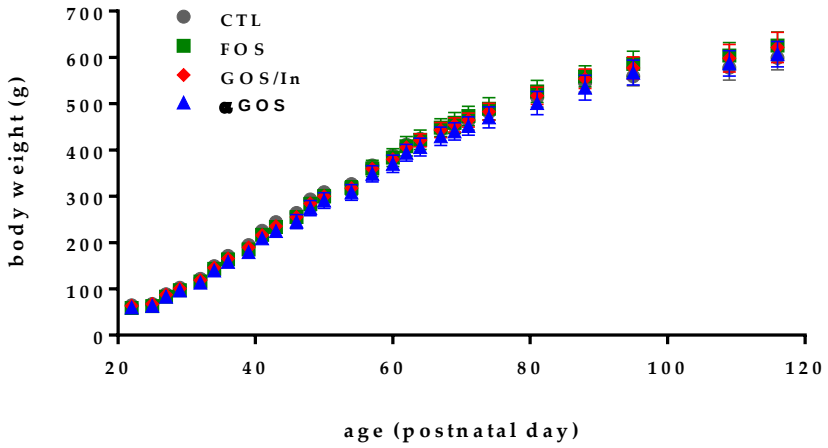

Figure S2. Growth of weaned rats until adulthood. Data are means +/- SD(n=8 per group).

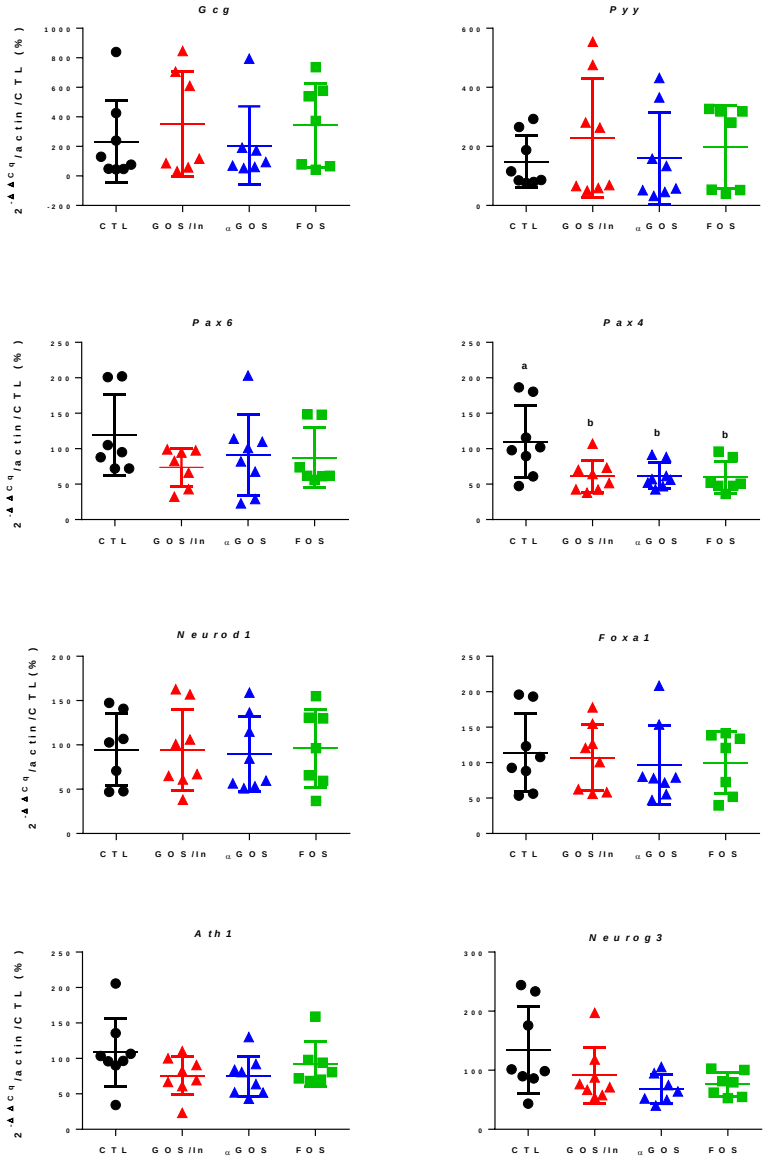

Figure S3. Relative expression of gene implied in the endocrine lineage and in L-cells differentiation in the colon. Different letters indicate significant difference between groups ($p < 0.05$). Data are fold-change expressed in % of CTL group. Individual, mean and SD values are plotted (n= 7 to 8 per group).

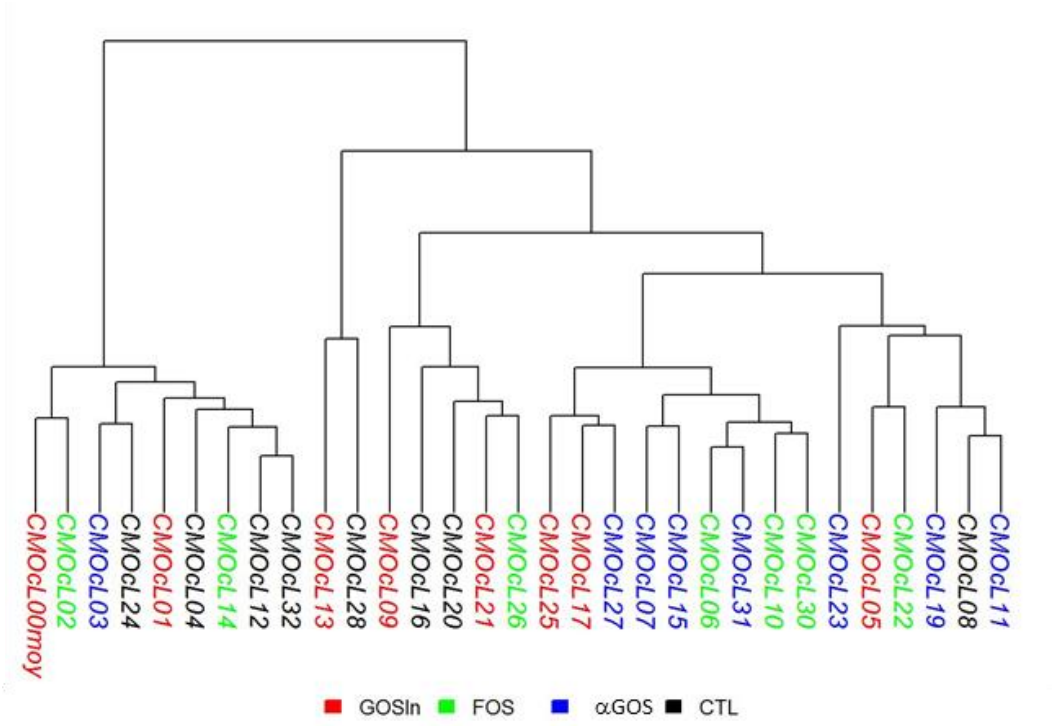

Figure S4. Hierarchical clustering based on the Ward's method of phylogenetically informed distance matrix computed using the unweighted UniFrac metric for cecocolonic contents collected at PND 124/126.

Table S2. Concentration of SCFA in ileal and ceco-colonic contents at PND 124/126

	Ileal concentrations (mM)			Ceco-colonic concentrations (mM)			
Treatment	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**	**Minors**
CTL	2.1 ± 1.0	0.1 ± 0.1	ND[1]	13.5 ± 3.3	3.5 ± 0.8	2.7 ± 1.1	1.4 ± 0.5
FOS	2.2 ± 1.6	0.1 ± 0.1	ND	11.1 ± 1.9	2.9 ± 0.5	2.1 ±0.7	1.2 ± 0.2
GOS/In	2.7 ± 1.5	0.2 ± 0.2	ND	11.7 ± 3.1	3.3 ± 0.9	1.9 ± 0.7	1.3 ± 0.5
αGOS	2.9 ± 1.4	0.1 ± 0.1	ND	10.7 ± 1.9	2.9 ± 0.5	1.7 ± 0.5	1.2 ± 0.3

[1] ND, non-detectable. Data are means ± SD (n=7 to 8 per groups)