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# Impact of infant nutrition on gut and brain nitrogenous metabolomes: Comparison of human milk and infant formula feeding in the minipiglet model

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**Abstract**

The effect of infant nutrition on the metabolism of different body compartments is poorly described. Hence, the present study aimed to characterize the effect of human milk (HM) *vs.* infant formula (IF) feeding on metabolic mediators in key samples crucial for metabolic activity through the gut-brain axis during infant development, using the minipiglet as a human infant model. Eighteen 19-day-old piglets were fed HM or IF for 6 days. Thirty min after the last meal, colonic digesta, blood plasma, liver and 6 regions of the brain were sampled. Profiles of 45 metabolites (including proteinous amino acids, tryptophan (Trp) metabolites, polyamines, neurotransmitters) were determined using a targeted liquid chromatography and tandem mass spectrometry approach. Metabolic signatures of key organs involved in the gut-brain signal exchange were diet-dependent. The main dietary-induced differences in metabolite content occurred in the hippocampus (77% of the targeted metabolites quantified), plasma (47%), brainstem (17%), and colonic digesta (16%). These differences concerned Trp, Trp-derived metabolites, polyamines, some proteinous amino acids and neurotransmitters. Tryptophan was preferentially metabolized towards the kynurenine pathway in the colon and the hippocampus of HM-fed piglets. Differences in brain amino acid levels were associated with different brain polyamine and neurotransmitter contents in the hippocampus and, to a lower extent, in the other brain regions. Significant ($P<.05$) correlations with specific bacterial genera and gene expressions were found. In the colon, Trp-derived metabolites such as kynurenine and tryptamine were positively and negatively correlated with *Veillonella*, respectively, and tryptamine levels may be related to the abundance of *Ruminococcus* genera. In the brain, the elevated level of the 5-hydroxyindolacetic acid (5-HIAA, from the serotonine pathway) in the HM brainstem may be related to the more abundant *Bifidobacterium* in HM-fed piglets. Finally, bacteria from the Firmicutes and Proteobacteria phyla may be involved in modulating polyamine production, as suggested by significant correlations between polyamine levels and bacterial genus abundances in the colon. Overall, the results confirmed the differential effect of HM *vs.* IF feeding on the microbiota-gut-brain axis and showed the high metabolic responsiveness of the hippocampus, probably related to specific nutritional needs and functionality in minipiglets.


*Keywords:* Human milk; Infant nutrition; Metabolome; Amino acids; Tryptophan; Hippocampus.

*Abbreviations:* AA, amino acid; HM, human milk; IF, infant formula; LNAA, large neutral amino acid; LOD, limit of detection; LOQ, limit of quantification; MFA, multifactor analysis; Trp, Tryptophan.

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† In memory of Dr Nicole C. Roy who played a key role in the present work.

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## 1. Introduction

Human milk (HM) is a complex fluid containing a myriad of bioactive compounds, providing the nutritional requirements of infants and ensuring their optimal growth and development [1–3]. However, a high rate of infants receiving infant formula (IF) is still worldwide reported [4]. Despite the optimization of IF manufacturing over the last few decades [5], differences in IF nutritional quality related to milk origin (e.g., bovine milk *vs*. HM) and IF manufacturing practice [6–8] still exist, with consequences on digestion kinetics [9–13], altogether leading to metabolic discrepancies between breastfed and IF-fed infants [14–26].

Although the underlying mechanisms of communication between the intestinal microbiota and the gut-brain axis are not fully understood, vagal, immune and endocrine pathways are associated with reciprocal exchanges of signals from the gut and the brain, and of metabolites produced by the intestinal microbiota [27,28]. Within this complex system of interactions, early postnatal life nutrition plays a central role *via* nutrient interaction with intestinal epithelial cells and microbiota, priming the balance between pathways, thereby modulating infant development. However, the dietary impact on metabolic signatures of key organs involved in the gut-brain signal exchange is poorly documented in this sensitive time window for infant development.

In a preclinical study, we have recently demonstrated that the microbiota-gut-brain axis development is modulated by infant nutrition [19]. The intestinal microbiota profile associated with HM-feeding correlated with an improved maturation of the intestinal epithelial barrier, immune system and endocrine functions, and with a modulation of intestinal and brain tryptophan (Trp) metabolism as well as with several other brain functions in the early postnatal period of life [19].

To better understand the underlying mechanisms of intercommunication between the intestine and brain regions, the present study aimed to compare metabolomic profiles of colonic digesta, plasma, liver and 6 regions of the brain, between HM- and IF-fed Yucatan minipiglets used as a human infant model. The suckling pig is a well-established and suitable animal model for human infants, with a digestive system that closely mimics that of humans and similar brain development [29]. The relationship between dietary protein reaching the gut microbiota and inducing the release of metabolites and their bioavailability in the brain has been demonstrated in the pig model [30–33]. In addition, the Yucatan minipig has the advantage of consuming less food (and therefore less milk) than the conventional pig and provides access to a sufficient amount of samples (tissue and digesta) for different analysis. A targeted metabolome approach using tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) was performed and allowed the quantification of 45 metabolites, including proteinous amino acids (AAs), Trp-derived metabolites, polyamines, neurotransmitters and other nitrogenous compounds. Correlations with our previously published data [19] were also carried out (gene expression and gut microbiota).

## 2. Materials and methods

### 2.1. Human milk collection and animal study

The protocol for HM collection was ethically approved by the Institutional Review Board of South Mediterranean V (n°19.12.12.65653). Two types of HM pools were used. For the first pool, HM samples from 22 donor mothers (range of women's lactation period: 0.30–5.61 months postpartum) obtained frozen from the donor milk bank of the Rennes University Hospital Center were heat treated according to Holder pasteurization (62.5°C, 30 min) prior to pooling to achieve the same crude protein, Trp and fat content as the IF fed (Table 1), and stored at −20°C until distribution. For the second pool, raw HM samples were collected from 15 to 20 healthy mothers within each experimental block (range of women's lactation period: 0.8–2.7 months postdelivery), pooled and stored at 4°C until use the day after collection. The animal study was designed and carried out at INRAE experimental facilities (UE1421 UE3P, Saint-Gilles, France; Agreement No. D35-275-32; doi: 10.15454/1.5573932732039927E12), in accordance with the current ethical standards of the European Community (Directive 2010/63/EU) and the French legislation on animal experimentation and ethics (authorization #2020020610329770) and the ethics committees of the CREEA (Rennes Committee of Ethics in Animal Experimentation) and the French Ministry of Higher Education. Animal welfare was monitored throughout the protocol by daily observation and weighing every 3 days. No medication or antibiotic treatment was delivered.

The animal study was conducted as described elsewhere [19] and a summary of the overall experimental design presented in Figure 1. Briefly, 18 individually housed 10-day-old Yucatan minipiglets (8 males, 10 females) were first fed with an adaptation diet for 8±2 days (adaptation diet based on full-fat raw cow milk powder enriched with vitamins and minerals) before being randomly assigned to 1 of the 2 dietary groups (HM *vs*. IF, experimental period). Allocation to each diet was balanced between groups according to BW at 10 days of age, litter origin and sex (4 males and 5 females per dietary group). Piglets of HM-group received the first pool of pasteurized HM for 5 days before being fed the second pool of raw mature HM on the last day before euthanasia. Piglets of IF-group received a rehydrated cow milk-based IF (115 g powder/L IF) as described previously [19] for 6 days. The diet compositions are detailed in Table 1. The AA concentration of the experimental diets, HM and IF, is given in Charton et al. [34], while the AA profiles of fresh HM and IF samples are presented in the Supplementary Fig. 1. Liquid vanilla was added to all diets (0.3 g/100 mL diet) to encourage intake during both the adaptation and experimental periods. Dietary intake was recorded at each meal. Throughout the entire experimental period, HM- and IF-piglets had no significant difference in food intake (average of 255±7 g/kg BW/day) or weight gain (54±5 g/day), such as reported in Charton et al. [19]. The experimental design for the HM and IF groups was conducted as 3 independent blocks ($n_{block\ 1}$=8, $n_{block\ 2}$=6, $n_{block\ 2}$=4).

### 2.2. Sample collection

On the last day of the experiment, the piglets received 6 hourly meals before being euthanized by electrical stunning 30 min after the last meal, immediately followed by exsanguination as described previously [19]. Blood was collected under Acid-Citrate-Dextrose (ACD)-anticoagulated tubes for plasma collection. Plasma was collected from blood samples after centrifugation (3000 g, 10 min, 4°C) and stored at −80°C until analysis. Immediately after sacrifice, the brain was extracted, and the 6 brain regions (pineal gland, brainstem, and 4 regions in the left hemisphere: hippocampus, hypothalamus, prefrontal cortex, and striatum) were sampled. Brain samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Colonic digesta within the first third of the colon and a piece of liver were collected and immediately frozen in liquid nitrogen before being stored at −80°C until analysis. Diets were also sampled and stored at −80°C until analysis.

### 2.3. LC-MS/MS analysis

Samples of precisely weighed tissues (~20 mg) and plasma (20 μL) were vortexed with ceramic beads and 80 μL ice-cold ace-

Table 1
Protein, tryptophan and lipid content of pasteurized human milk (PHM), raw human milk (RHM) and the infant formula (IF) (mean ± SEM).

| g/100 mL | PHM* | RHM† | IF | *P*-value | | |
|---|---|---|---|---|---|---|
| | | | | RHM *vs.* PHM | RHM *vs.* IF | PHM *vs.* IF |
| Crude protein‡ | 1.67±0.07 | 1.37±0.07 | 1.44±0.02 | .025 | .403 | .014 |
| True protein§ | 1.28±0.05 | 0.95±0.05 | 1.37±0.02 | .003 | .001 | .085 |
| Tryptophan | 0.03±0.001 | 0.02±0.001 | 0.03±0.001 | .002 | .010 | .339 |
| Lipids | 3.21±0.04 | 2.79±0.04 | 3.15±0.06 | .050 | .008 | .714 |

* Average values of pasteurized human milk. The PHM represents a pool of 22 anonymous donations of human milk heat-treated by Holder pasteurization to reach a similar crude protein and tryptophan composition as the Infant formula composition. These milks were obtained frozen from the donor milk bank of the Rennes University Hospital Centre (France).
† Average values of raw human milk. The RHM represents a pool of 20-25 anonymous volunteer donations of human milk without any heat treatment applied. The volunteers' recruitment was in partnership with the nursery of the hospital of Rennes.
‡ Crude protein = Total nitrogen * 6.38.
§ True protein = Crude protein * (1-%NPN)PHM, pasteurized human; milk; RHM, raw human milk; IF, bovine-milk-based infant formula.

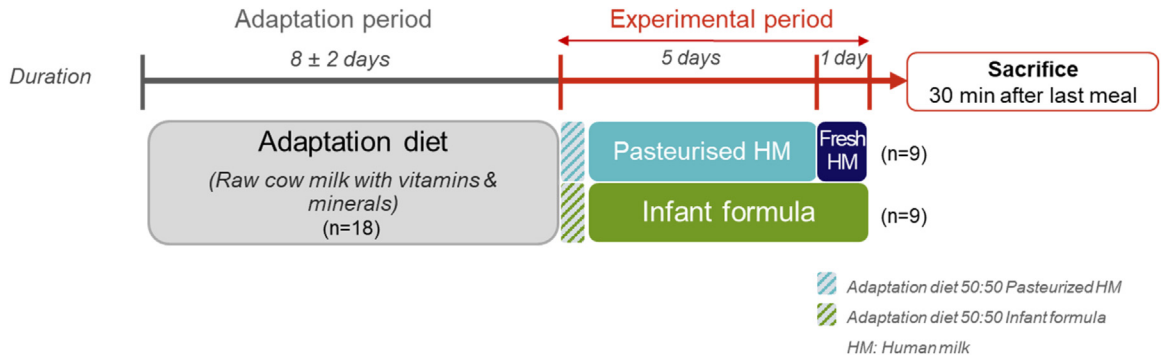


**Fig. 1.** Overview of the experimental study design.

tonitrile prior to vertical mixing using a Qiagen Tissue Lyser II (20 rotations/s, 1 min) for protein precipitation. Mixed samples were then degassed in a 0°C ultrasound water bath for 10 min prior to being beat shake (20 rotations/s, 1 min), vortexed and centrifuged (12350 g, 10 min, 4°C). Supernatants (20 µL) were derivatized, as described previously [35], by sequential addition of 10 µL of 100 mM sodium carbonate, 10 µL of fresh $^{12}$C benzoyl chloride solution (2% (v/v) in acetonitrile, Toronto Research Chemicals, Toronto, Canada) and 10 µL of the internal standard. The mixture was diluted with 50 µL of ultrapure water (Milli-Q® water, Millipore, Bedford, MA, USA) and homogenized prior to analysis on LC-MS/MS.

The internal standard solution was composed of a mixture of the targeted metabolites analyzed (Supplementary Table 1) derivatized by $^{13}$C-benzoyl chloride solution (2% (v/v) in acetonitrile (Toronto Research Chemicals, Toronto, Canada).

The Exion LC UHPLC unit system (AB SCIEX LLC, CA, USA) equipped with an ACQUITY UPLC HSS T3 VanGuard™ pre-column (1.8 µm, 2.1×5 mm, Waters, Ireland) coupled to an ACQUITY UPLC HSS T3 column (1.8 µm, 1.0×100 mm, Waters, Ireland) stored at 27°C, was used for liquid chromatography. Two µL of spiked samples stored at room temperature in the autosampler were injected into the column for analysis. Samples were separated over 20 min using a binary gradient with the mobile phase, including ammonium formate with 0.1% formic acid (solvent A) and acetonitrile (100%, solvent B). The flow rate was 100 µL/min, and the elution gradient program was as follows: 0 min, 100% A; 0.5 min, 85% A; 14 min, 83% A; 14.5 min, 45% A; 18 min, 30% A; 19 min, 0% A; and 20 min, 100% A. An additional 10 min of column equilibration at 100% A was required to achieve reproducible chromatography.

The mass spectrometry data were acquired using a SCIEX 6500+ Triple Quad MS/MS system equipped with an electrospray ionization source operated in positive ion mode at 4.5 kV and in dynamic multiple reaction monitoring (dMRM). The gas temperatures of the 2 ion sources were 45°C and 70°C, respectively, the gas flow was 11 L/min, and the nebulizer pressure was 25 psi. Nitrogen gas was used for nebulization, desolvation, and collision. The transition settings are given in Supplementary Table 2.

The metabolic approach was used to identify and quantify 45 targeted metabolites using stable-isotope internal standards, including free proteinous AAs, Trp-derived metabolites, polyamines and derivatives, neurotransmitters including dopamine-derived metabolites, other metabolites such as arginine-derived metabolites, antioxidant peptides or low abundant amines, such as described in Figure 2. Of the AA analyzed, arginine was not measured as the arginine peak eluded with that of another metabolite.

Automated peak integration was performed using MultiQuant software (version 3.0.3). After visual inspection of all peaks to ensure an accurate integration, automated quantitation was performed based on the ratio of the integrated peak area of targeted metabolites to that of the isotopically-labelled internal standards. The obtained concentrations were then compared to the limits of detection (LOD) and limits of quantification (LOQ). Concentration values below LOD are considered with a small value of 0.0001, and concentration values below LOQ are considered as 50% of LOQ. Metabolite concentrations were finally corrected by the dilution factor arising from the sample preparation and the dry matter (DM) sample content (for the colon) and expressed as µmol/kg DM. The full raw data of metabolite concentrations determined in all samples of HM and IF diets, and in all samples of plasma, liver,

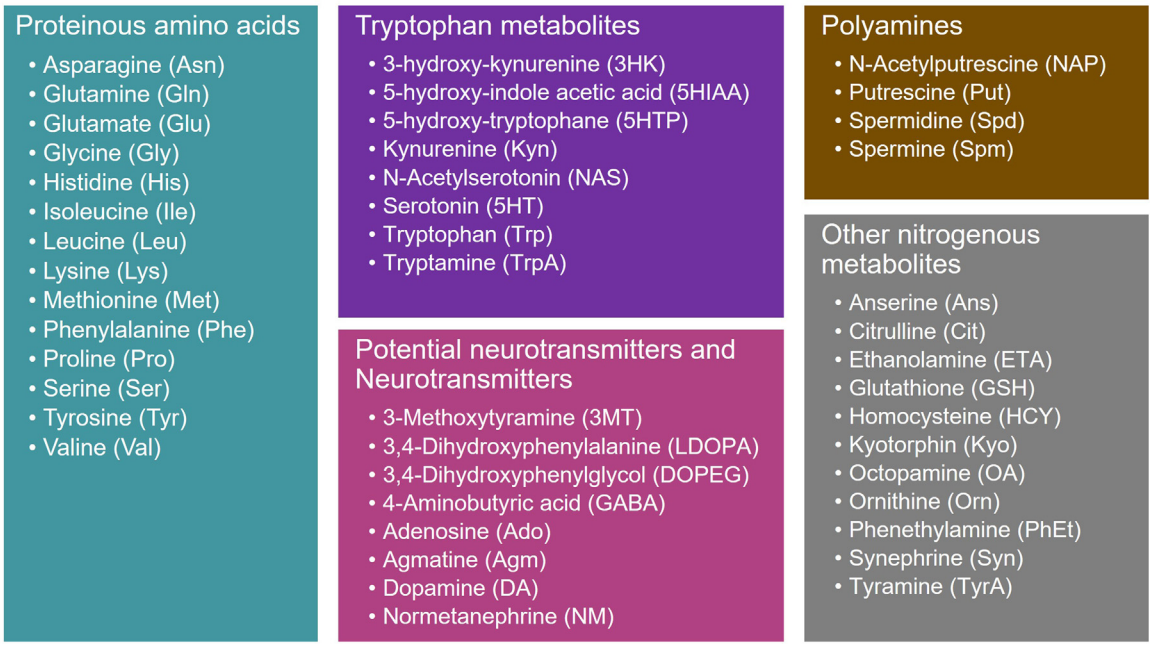


**Fig. 2.** Classes of targeted metabolites quantified by LC-MS/MS.

colonic digesta and 6 brain areas of HM- and IF-fed piglets are available in an online dataverse https://doi.org/10.57745/ZVWTVX.

### 2.4. Statistical analyses

Statistical analyses were conducted using R software (version 3.6.2 [36]).

#### 2.4.1. Dataset processing

Prior to conducting the statistical analysis, the dataset was visually inspected by site of interest to remove metabolites with a high number of missing data ($n_{\text{quantifiable content}} \geq 3$ for the liver and $n_{\text{quantifiable content}} \geq 4$ for the other sites). Outlier piglet samples were identified by site and corresponded to samples having more than 20% of metabolite concentrations defined as extreme outlier values (*i.e.* > third quartile + 3 * interquartile range or < first quartile – 3 * interquartile range, 'rstatix' package [37]) and/or to samples having more than 40% of metabolite concentrations under the LOD or LOQ) [38]. Such outlier piglet sample values were removed from the final dataset.

#### 2.4.2. Unidimensional analysis

A linear model was used to test the significance of the following fixed effects: diet, block, and sex on each metabolite concentration by site of interest (colon, liver, plasma, 6 brain regions). When block or sex effects were nonsignificant, they were removed from the linear model. The normal distribution and the homoscedasticity of the residuals of each linear model were tested using Shapiro–Wilk and Levene's tests, respectively. When the raw data did not satisfy these assumptions, a natural logarithmic data transformation was performed before running the linear model. If the assumptions were still not fulfilled, data were tested using a nonparametric Wilcoxon test. Differences were considered statistically significant at $P<.05$, without correction for false discovery rate. Data are presented as means ± standard error of the mean (SEM).

#### 2.4.3. Multidimensional analysis

A first multiple factor analysis (MFA, 'FactomineR' package [39,40]) was performed on all the metabolite concentrations grouped by sampling site (brainstem, hippocampus, hypothalamus, pineal gland, prefrontal cortex, striatum, colon, liver and plasma) and metabolite family (AAs, Trp and Trp-derived metabolites, neurotransmitters, polyamines and other nitrogenous analytes (arginine-derived metabolites, trace amines, antioxidant peptides, kyotorphin and ethanolamine)), resulting in 45 different groups gathering 306 variables.

To provide a deeper insight into the metabolic signals between the gut microbiota, the intestine and brain regions, a second MFA was performed based on the present metabolite data grouped by sampling site and metabolite family, as described above, and on previously published [19] gut microbiota variables (bacterial genus abundances in ileum and colon) grouped according to their respective phylum, and intestinal epithelium variables (morphometry, gene expression, goblet cell counting, GLP1 content and cell numbers determined in ileum and colon) grouped according to their functions (5 functions: barrier, immune and endocrine functions, Trp metabolism, nutrient carrier/digestion). Brain variables (gene expression determined in the 4 following brain regions: hippocampus, hypothalamus, prefrontal-cortex, striatum) were grouped according to 7 functions (functions: genes encoding in barrier, immune and endocrine functions, Trp metabolism, nutrient carriers/digestion, neurotransmitters, neurosynaptogenesis), previously published [19]. This MFA resulted in 90 groups gathering 930 variables.

Concerning these 2 above-mentioned MFAs (the first MFA (MFA1) performed with metabolites alone; the second MFA (MFA2) performed with metabolites plus gut microbiota, intestinal and brain variables), the variables that significantly contributed to the separation of HM- and IF-piglets were available on an online dataverse: https://doi.org/10.57745/ZVWTVX.

#### 2.4.4. Data correlation

Pearson correlation coefficients were determined between (1) colonic metabolites and previously published colonic microbiota

data [19], [2] colonic metabolites and colonic variables (morphometry, gene expression, goblet cell counting, GLP1 content and cell number) previously published [19]; (3) brain metabolites and previously published gene expression [19] in the hippocampus, hypothalamus, prefrontal cortex and striatum. Correlations were determined regardless to the diet-group. Correlations were significant when $P<.05$ and $|r|\geq 0.5$. The full data set is available on an online dataverse: https://doi.org/10.57745/ZVWTVX.

## 3. Results

An average of 76±2% of the targeted metabolites were quantifiable in at least 1 site sampled (Supplementary Table 3) and were retained for statistical analysis.

### 3.1. Overall impact of HM vs. IF feeding on the colon, liver, plasma and brain metabolome profiles

The effect of diet on metabolic profiles was dependent on the sites sampled, with a greater number of metabolites with dietary differences ($P<.05$) found in the hippocampus (77% of the metabolites analyzed significantly different between HM-fed and IF-fed, Fig. 3D) and plasma (47%, Fig. 3C), and, to a lower extent, in the brainstem (17%, Fig. 3E), colon (16%, Fig. 3A), prefrontal cortex (15%, Fig. 3F), liver (10%, Fig. 3B), striatum (6%, Fig. 3G), and hypothalamus (6%, Fig. 3H). There was no significant difference between diets for the pineal gland (0%, Fig. 3I). Overall, the effect of diet ($P<.05$) mainly concerned AAs (41% of the metabolites over all the sites sampled) and, to a lesser extent, polyamines (17%), Trp-derived metabolites (14%) and neurotransmitters (11%). Metabolites involved in dopamine metabolism, the urea cycle, trace amines, antioxidant peptides and other nitrogenous metabolites were less affected ($P<.05$) by diet (only 2 to 6% of the metabolites were significantly different between the dietary groups).

The first MFA aimed to analyze the overall relationship between the dietary groups and the metabolites gathered by sampling site ($n=9$) and metabolite type ($n=5$), leading to 45 groups of variables. This analysis revealed that some site-specific metabolite profiles discriminated the dietary groups of piglets on Dimension 2, explaining 14% of the variance (Fig. 4). Among all the groups of variables included in this MFA, 19 of them significantly contributed to Dimension 2. Dimension 2 was mainly explained by the 5 metabolite families in the liver, the hippocamus and the brainstem (except the AA group for the latter 2 groups), by the Trp-derived metabolites group in colon and plasma and by the AA group in plasma.

#### 3.1.1. Amino acids and tryptophan-derived metabolites

The levels of several AAs (Asparagine, Asn; Glutamine, Gln; Glycine, Gly; Leucine, Leu; Lysine, Lys; Methionine, Met; Phenylalanine, Phe; Trp; Tyrosine, Tyr; Valine, Val) were significantly lower in the plasma of HM-fed than IF-fed piglets (Fig. 3C, Supplementary Table 3). In contrast, the levels of these AAs were significantly higher in the hippocampus of HM-fed than IF-fed piglets, except for Met and Tyr, where the concentration differences did not reach statistical significance (Fig. 3D, Supplementary Table 3). In addition, the levels of other AAs, such as Glutamate (Glu), Histidine (His), Isoleucine (Ile), Proline (Pro) and Serine (Ser), were also significantly higher in the hippocampus of HM-fed than IF-fed piglets.

Only a few differences were found on the other sites (Fig. 3, Supplementary Table 3). Glu, and Met were significantly less concentrated in the colon of HM-fed piglets than in that of IF-fed piglets, as observed for Met in the striatum. The level of Pro was significantly higher in the colon and liver and that of Trp (tendency) in the liver of HM-fed piglets compared to those of IF-fed piglets.

Tissue and plasma levels of several Trp-derived metabolites were higher in HM-fed than IF-fed piglets. The levels of the targeted metabolites involved in the kynurenine pathway, kynurenine and 3-hydroxykynurenine (3-HK), were significantly higher in the colon and hippocampus, respectively, of HM-fed piglets than in those of IF-fed piglets (Fig. 3A and D, Supplementary Table 3). Tryptamine was significantly less concentrated in the colon of HM-fed piglets, while it was significantly more concentrated in the hippocampus of HM-fed piglets than that of IF-fed piglets. The 5-hydroxyindolacetic acid (5-HIAA), a Trp-derived metabolite of the serotonin pathway, was more concentrated in the brainstem of HM-fed than IF-fed piglets (Fig. 3E, Supplementary Table 3).

#### 3.1.2. Ornithine and derived polyamines

Ornithine concentration was significantly higher in the hippocampus of HM-fed than IF-fed piglets (Fig. 3D, Supplementary Table 3). The concentration of polyamine metabolites (N-acetylputrescine (NAP), putrescine, spermidine and spermine) was significantly affected by diet type. In colonic digesta, spermidine was significantly less concentrated in HM-fed than IF-fed piglets (Fig. 3A, Supplementary Table 3). In the hippocampus, the levels of ornithine and derived polyamines were significantly higher in HM-fed than IF-fed piglets. Regarding the other sites, putrescine was also significantly more concentrated in the brainstem, hypothalamus, prefrontal cortex, and plasma of HM-fed than IF-fed piglets (Fig. 3, Supplementary Table 3). NAP and spermidine were significantly more concentrated in the brainstem and liver, respectively, of HM-fed than in those of IF-fed piglets.

#### 3.1.3. Neurotransmitters, including dopamine-derived metabolites

The level of agmatine was significantly higher in the brainstem, hippocampus and prefrontal cortex of HM-fed than IF-fed piglets (Fig. 3, Supplementary Table 3). Similarly, adenosine was significantly more concentrated in the liver and the prefrontal cortex of HM-fed than IF-fed piglets, such as 4-aminobutyric acid (GABA) in the HM-fed hippocampus.

Metabolites involved in dopamine metabolism were dopamine (DA), 3,4-dihydroxyphenylalanine (L-DOPA), and 3,4-dihydroxyphenylglycol (DOPEG). Plasma DA and DOPEG levels were significantly higher in HM-fed than IF-fed piglets (Fig. 3C, Supplementary Table 3). Whereas L-DOPA was significantly less concentrated in the striatum, a significantly higher level of DOPEG was measured in the brainstem of HM-fed than IF-fed piglets (Fig. 3E and G, Supplementary Table 3).

### 3.2. Overall impact of the diet on the total microbiota-gut-brain axis

In order to gain a deeper insight into the metabolic signals between the gut microbiota, the intestine and the brain regions, a second MFA (Fig. 5) was performed on 930 variables, including the present data on metabolites and the previously published data [19], distributed in 90 groups. Additional correlation analyses (Figs. 6 and 7) were performed in the colon between genes, microbiota genera and metabolites, and by site between genes and metabolites in various brain regions, regardless to diet to provide further knowledge on the interactions between metabolite signals and gene expression or microbiota composition within brain or colon sites.

The second MFA presented in Figure 5 aimed to analyze the relationship between the dietary groups and variables gathered by sampling site and their related function. This analysis revealed that HM-fed and IF-fed piglets were mainly discriminated on dimension 1, explaining 12.7% of the variance (Fig. 5). Among all the groups of variables included, 46.7% significantly contributed (% of significative contribution $>$ 1.1%) to dimension 1. The major groups of

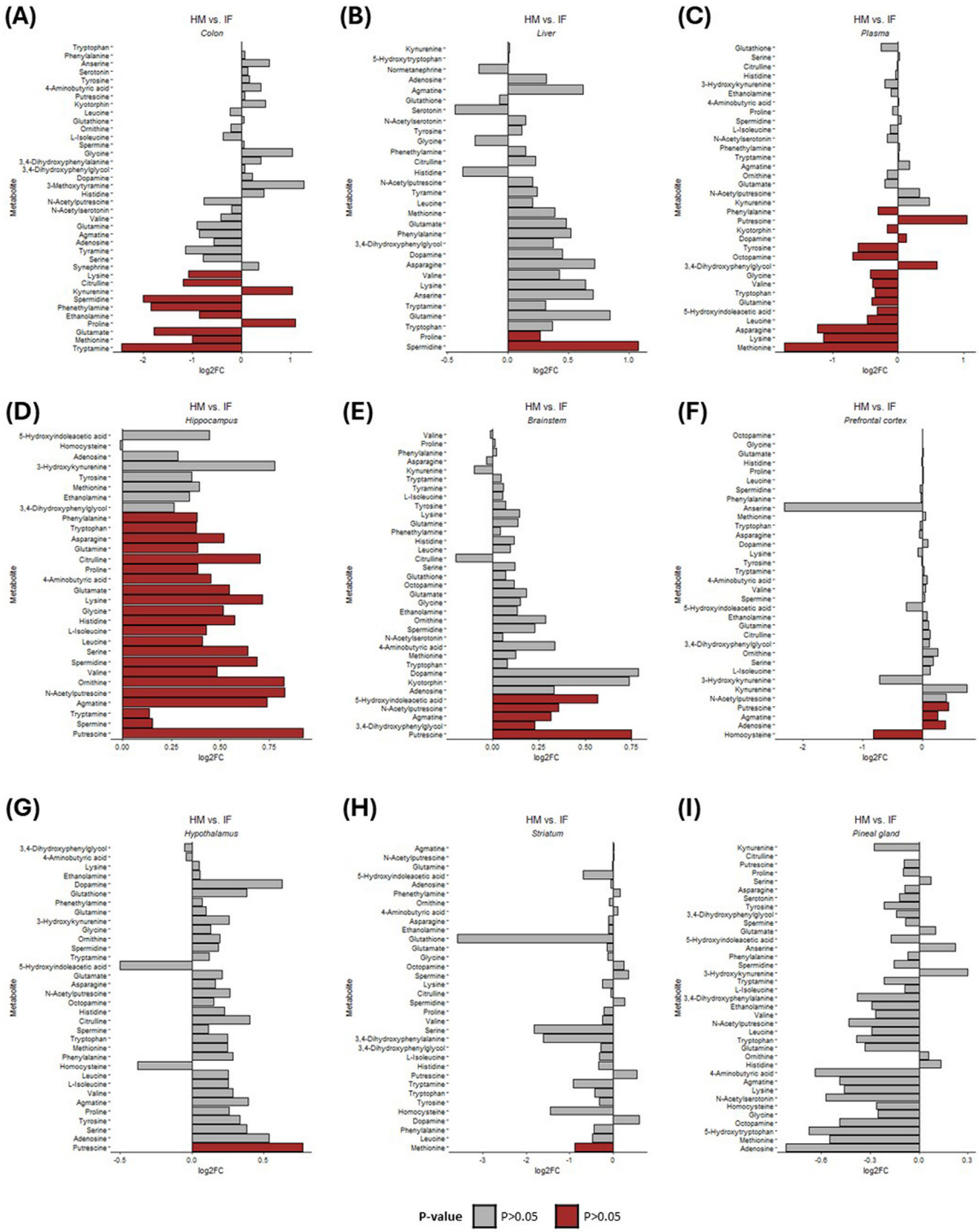


**Fig. 3.** Differential concentrations (log2FC) of metabolites between HM- and IF-fed piglets in the 9 sites sampled: (A) Colon; (B) Liver; (C) Plasma; (D) Hippocampus; (E) Brainstem; (F) Prefrontal cortex; (G) Hypothalamus; (H) Striatum; (I) Pineal gland. Metabolites are sorted according to the statistical significance of their differential concentration between HM and IF groups. Red filling means a significant differential concentration between HM and IF groups ($P<.05$). The final dataset was composed of metabolite concentrations from 6 piglets by dietary group for the liver, 8 piglets by dietary group for the colon, 9 piglets by dietary group for the plasma, brainstem, hippocampus, hypothalamus, and prefrontal cortex, and 8 HM-fed piglets and 9 IF-fed piglets for the striatum and pineal gland, respectively.

variables discriminating the 2 dietary groups were found in the colon (19.0%) and striatum (14.3%). The groups of variables from the ileum, hippocampus and prefrontal cortex (11.9%), hypothalamus (9.5%), as well as liver and microbiota (7.1%) and plasma (4.8%), contributed to the dietary group discrimination to a lesser extent, while brainstem and pineal gland variables did not contribute.

Regarding the groups of variables including gene expression and metabolite variables, the main groups of variables significantly contributing to the description of Dimension 1 were related to Trp metabolism at the intestinal, brain and plasma levels (16.7% of variable group contribution), to neurotransmitters in the brain and plasma (14.3% of variable group contribution), and to the group of proteinous AAs in

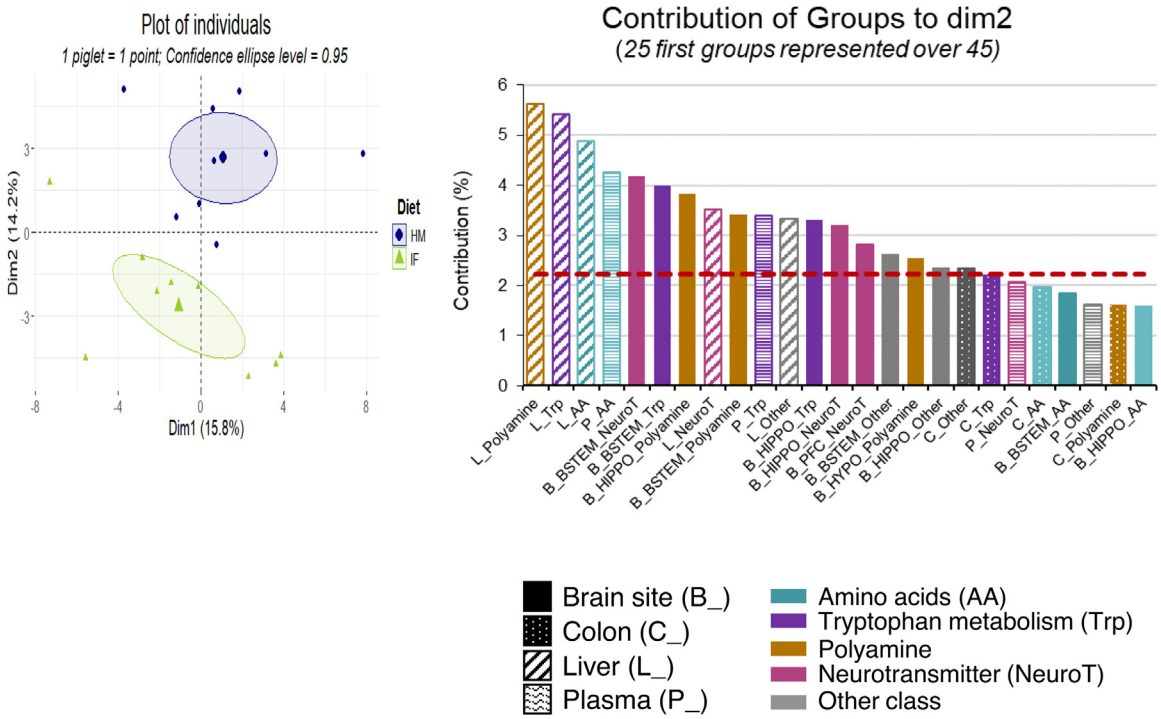


**Fig. 4.** Multiple factor analysis (MFA) with metabolite concentrations from HM- and IF-piglets, grouped by site and metabolite family (detailed information on Fig. 2) ($n$=45 groups of variables). Red dot line: level of statistical significance corresponding to the inverse of the number of variable groups used in the analysis (base 100 divided by the total number of groups or variables). B.BSTEM: Brainstem; C: colon; B.HIPPO: Hippocampus; B.HYPO: Hypothalamus; L: Liver; P: Plasma; B.PFC: Prefrontal cortex; B.PNGL: Pineal gland. Group labels are composed of the Site_metabolite family.

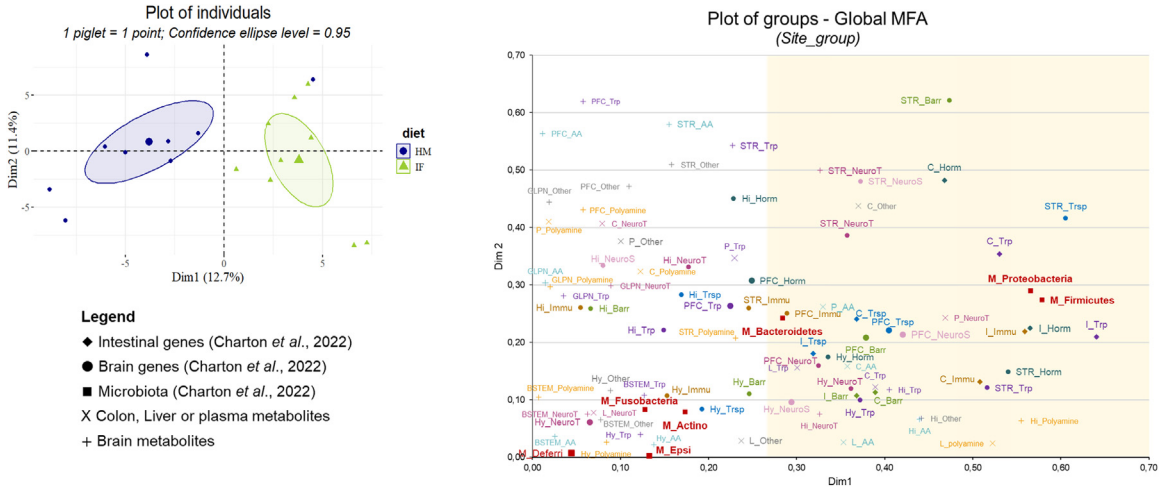


**Fig. 5.** Multiple factor analysis (MFA) with groups of variables including the present families of metabolites and the previously published data by Charton et al. [19] ($n$=90 groups with 10 sites sampled (ileum, colon, liver, plasma, brainstem, hippocampus, hypothalamus, pineal gland, prefrontal cortex, striatum), 10 groups of variables grouped into functions (Transport/Nutrient carriers, barrier, endocrine and immune functions, tryptophan metabolism, neurotransmitters, neurosynaptogenesis, proteinous AAs, polyamines and other metabolites) and 5 groups of bacterial genera grouped into 5 microbiota phyla). The symbols represent each of the dataset. Groups names were written as Site_related function for published data [19] and metabolites as Site_family of metabolites. Groups significantly contributing to defining Dimension 1 are in the yellow area. AA: amino acids; Actino: Actinobacteria; Barr: barrier function; BSTEM: brainstem; C: colon; Deferri: Deferribacteres; Epsi: Epsilonbacteraeota; Hi: hippocampus; Horm: Hormones/endocrine function; Hy: hypothalamus; I: ileum; Immu: Immune function; L: liver; M: Microbiota; NeuroS: neurosynaptogenesis; NeuroT: neurotransmitters; P: plasma; PFC: prefrontal cortex; GLPN: pineal gland; STR: striatum; Trp: tryptophan pathways; Trsp: Transport/Nutrient carriers; the full name of abbreviated metabolite names is given in Figure 2.

colon, plasma, liver and hippocampus (9.5% of variable group contribution).

Most of the analyzed metabolites in colonic digesta (97%) were significantly ($|r|\geq 0.5$, $P<.05$) correlated with the expression of several colonic genes (Fig. 6A) and/or microbiota genera (Fig. 6). Similarly, all metabolites analyzed in the striatum were significantly ($P<.05$) correlated with the expression of several striatal genes (Fig. 7A). In contrast, a low number of correlations was observed between metabolites and genes in the hypothalamus (38% of the analyzed metabolites, Fig. 7B), hippocampus (50% of the analyzed metabolites, Fig. 7C) and prefrontal cortex (12% of the analyzed metabolites, Fig. 7D).

Correlations were observed between Trp-derived metabolites and genes encoding immune and barrier functions and Trp

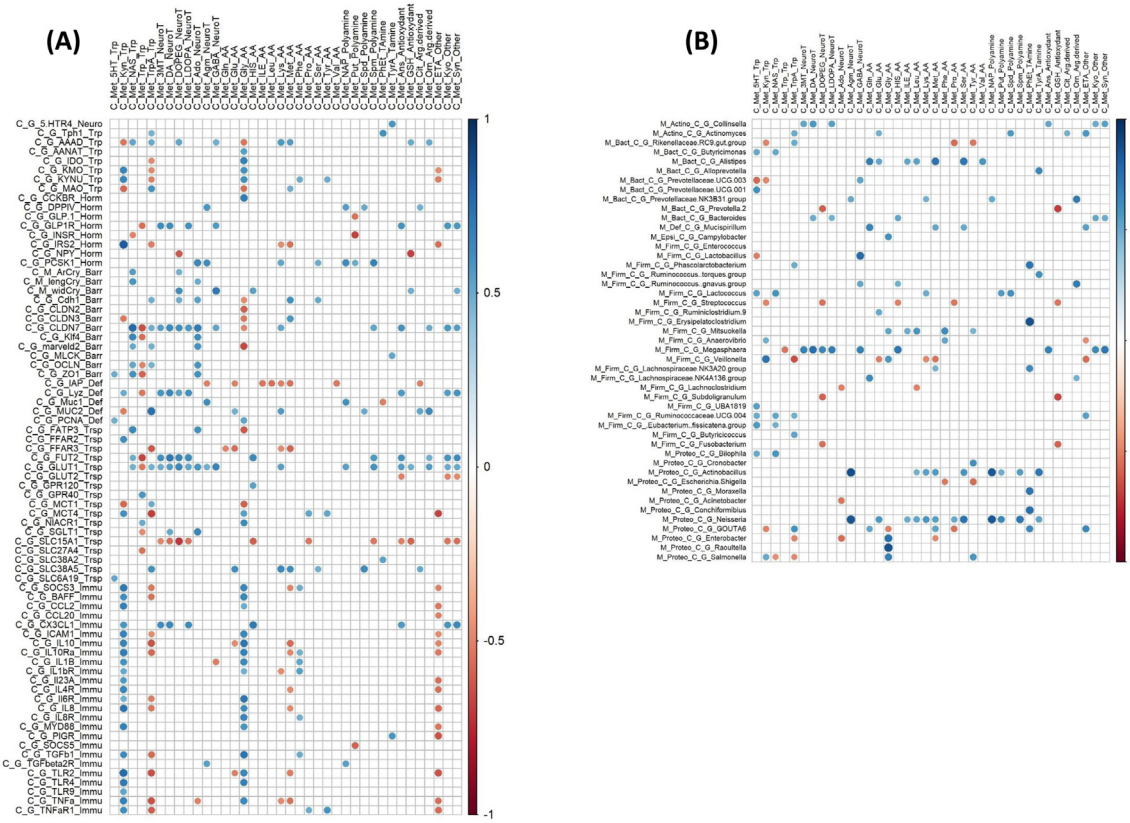


**Fig. 6.** Correlation matrix between colonic digesta metabolites and A, previously published colonic tissue gene expression [19] or B, colonic microbiota relative abundances [19] ($|r| \geq 0.5$, $P < .05$). C: colon; AA: Amino acid; Bact: Bacteroidetes; Barr: Barrier function; Def: Deferribacteres; Epsi: Epsilonbacteraeota; Firm: Firmicutes; Horm: Hormones/endocrine function; Immu: Immune function; NeuroT: neurotransmitter; Proteo: Proteobacteria; Trp: Tryptophan pathways; Trsp: Transport-Nutrient carrier; the full name of abbreviated metabolite names is given in Figure 2.

metabolism in the colon and brain regions, and between Trp-derived metabolites and genes encoding endocrine function, neurotransmitters and genes involved in neurosynaptogenesis in the brain regions (Figs. 6A and 7A-D). Correlations were also observed between neurotransmitters and genes encoding barrier, endocrine and nutrient carrier/transport functions in the colon, striatum and hypothalamus (Figs. 6A and 7A and B). For the proteinous AAs, several correlations were reported for Gly in the colon, for Glu and Ile in the hippocampus and for many AAs in the striatum with genes encoding most of the targeted functions (Figs. 6A and 7A and C). Finally, the concentration of several proteinous AAs in the colon was particularly correlated with the genera Alistipes (Bacteroidetes phylum) and Neisseria (Proteobacteria phylum), whereas the correlations for the other groups of metabolites with bacterial genera were metabolite-dependent (Fig. 6B).

## 4. Discussion

The metabolic signatures of key organs and metabolome pools involved in the gut-brain signal exchange are clearly impacted by the infant diet (HM *vs.* IF) in the minipiglet used as a human infant model. Compared to IF, HM induced differences in the levels of AAs, Trp and its derived metabolites, polyamines, and neurotransmitters and these differences varied with the site sampled. The levels of many metabolites were lower in the colon digesta and plasma but higher in the liver, hippocampus, and brainstem of HM-fed than IF-fed piglets. Only the levels of a few metabolites in the prefrontal cortex, striatum and hypothalamus differed according to the diet, with no difference observed in the pineal gland. Correlations highlight that the infant diet-induced modulation of gut microbiota and intestinal and brain gene expression [19] results in specific effects on colonic, plasma and brain metabolic profiles, particularly regarding Trp-metabolic pathways and AA and neurotransmitter levels.

### *4.1. Plasma and hippocampus amino acid levels were diet-dependent*

The levels of the essential AAs (except His and Ile) and several nonessential AAs were lower in the plasma of HM-fed than IF-fed piglets. This result agrees with previously reported plasma AA levels for breastfed and IF-fed infants during their first weeks [41,42] and months of life [43–48]. Some discrepancies exist between the present results and the literature data, however, regarding the impact of HM *vs.* IF on specific AAs, such as for plasma Met and Leu levels, which have not been previously reported as different between HM and IF-fed infants [42,44]. This difference may be due to the different experimental conditions, including different HM and IF composition and different *in vivo* models, despite the adequacy of the piglet for mimicking infant physiology [30–32].

Interestingly, the levels of all essential AAs and most of the nonessential AAs were higher in the hippocampus of HM-fed than IF-fed piglets. These results imply increased blood-brain AA transport but no measurement of these transporters in the brain was carried out in our study. It is known that AAs enter the brain as rapidly as glucose and are transported into the brain by the L-amino acid transporter 1 (LAT1 alias SLC7A5) [49]. Small neutral AAs, such as Gly and Pro, and GABA that were more concentrated in the HM-fed hippocampus, are markedly restricted in their entry

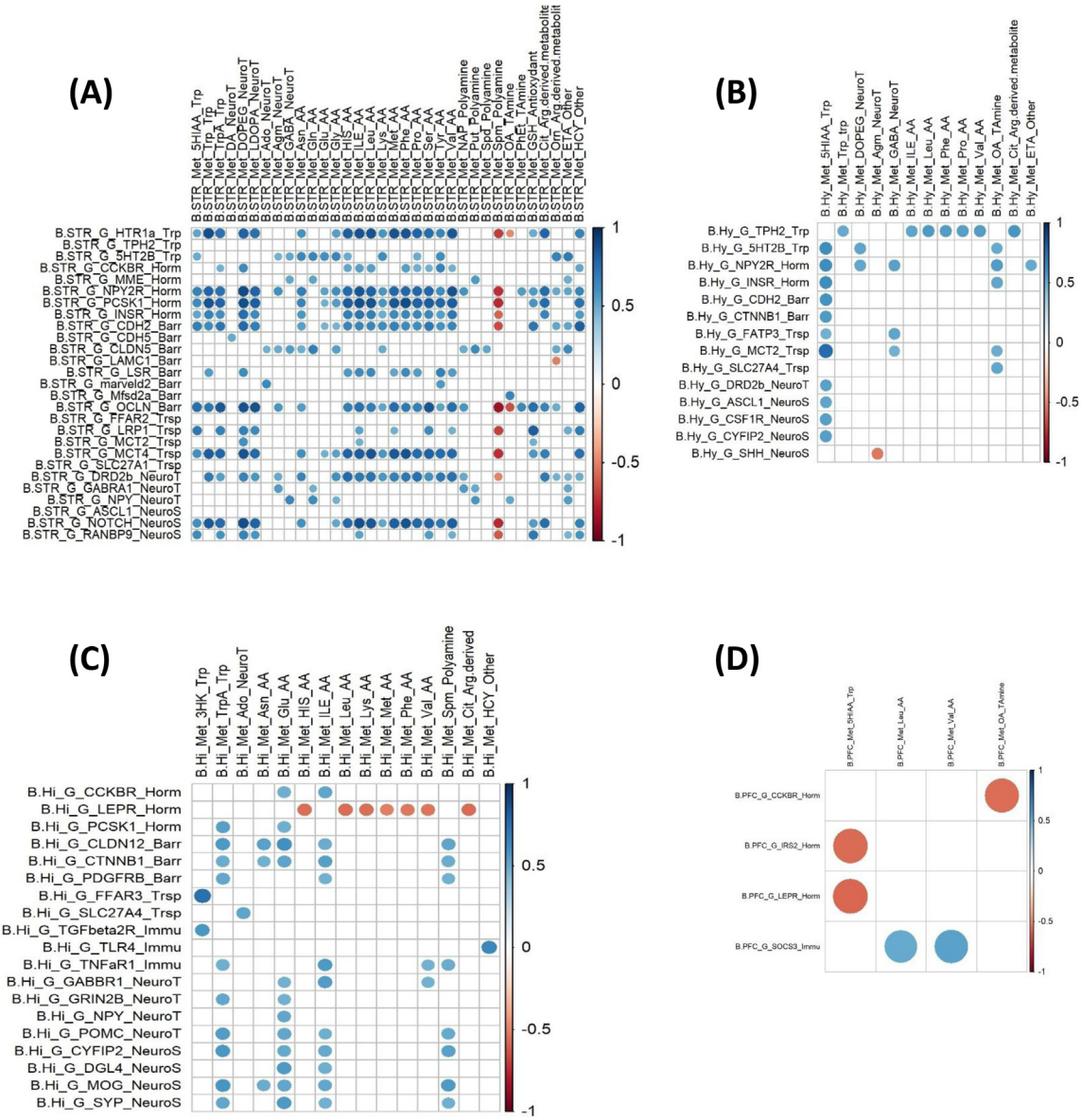


**Fig. 7.** Correlation matrix between metabolites and gene expression [19] in the striatum (A), the hypothalamus (B), the hippocampus (C) and the prefrontal cortex (D) ($|r| \geq 0.5$, $P < .05$). B.STR: Striatum; B.Hy: Hypothalamus; B.Hi: Hippocampus; B.PFC: Prefrontal cortex; AA: Amino acid; Barr: Barrier function; Horm: Hormones/endocrine function; Immu: Immune function; NeuroS: neurosynaptogenesis; NeuroT: neurotransmitter; Trp: Tryptophan pathways; Trsp: Transport-Nutrient carrier; the full name of abbreviated metabolite names is given in Figure 2.

into the brain via SLC6 transporter [50,51]. Therefore, the supply of AAs to the hippocampus seems to be prioritized over other brain regions in the piglet, and probably in the breastfed infant as well. The hippocampus is one of the regions of the brain that is the most strongly associated with cognitive function in early life [52]. Thus, the higher AA levels in the hippocampus could be attributed to a greater need for its cognitive functions.

### *4.2. Tryptophan was differently metabolized in response to the infant diet*

The signature related to the Trp metabolism (Trp-derived metabolites and genes) contributed significantly to the discrimination of HM and IF intestinal and brain tissues. For Trp, no difference in terms of concentration was observed in the colonic digesta, which is consistent with its similar true ileal and colonic digestibility reported between HM and IF [34]. The HM feeding led to a preferential metabolism of Trp into the intestinal kynurenine pathway compared to IF feeding, in accordance with the gene expression data, which showed a higher colonic expression of IDO (Indoleamine 2,3-Dioxygenase 1) and KYNU (Kynureninase) genes involved in the kynurenine pathway and a lower expression of Tph1 (Tryptophan hydroxylase 1), AAAD (Aromatic amino acid decarboxylase) and MAO (Monoamine Oxydase) genes involved in serotonin pathway in HM-fed piglets [19]. Consistently, higher urinary kynurenic acid levels were reported in piglets fed HM for 3 weeks than in milk formula-fed piglets [53]. Consistent with these diet-induced differences in Trp-derived metabolites in colonic digesta and gene expression in colonic tissue, positive correlations were observed between kynurenine and genes associated with KMO (Kynurenine 3-monooxygenase) and KYNU, and negative correlations were observed between kynurenine and genes associated

with AAAD and MAO in the colon. In addition, significantly lower levels of tryptamine and negative correlations between tryptamine and IDO, KMO and KYNU were found in the colonic digesta of HM-fed piglets compared to IF-fed piglets. The positive correlations of kynurenine with 80% of the targeted intestinal genes involved in immune functions, whereas tryptamine was negatively correlated with half of them, highlighted the important role of kynurenine metabolites in regulating intestinal immunity, which is beneficial for immune homeostasis [54,55]. However, these results should be interpreted with caution because metabolite concentrations were evaluated in the colonic digesta while gene expression was evaluated in colonic tissue. These differences in colonic Trp metabolic pathways may be partly explained by the colonic microbiota, consistent with the modulatory role of the microbiota on the host immune system mediated by Trp metabolism [54,55]. Indeed, kynurenine was positively and tryptamine negatively correlated with *Veillonella*, a genus more abundant in HM-fed piglets than IF-fed piglets, in agreement with previous data [19,56]. In addition, the higher tryptamine levels in IF-fed piglets compared to HM-fed piglets may be related to the higher abundance of *Ruminococcus* genera that can metabolize tryptophan to tryptamine [19,57–59]. In a large cohort of allergic infants, microbiota-dependent limitation of the abundance of trace amines (tryptamine, tyramine and phenylethylamine) was associated with the promotion of a tolerogenic immune development in infancy [60]. When AAs are fermentated by gut bacteria, they can also be converted to several other compounds, such as ammonia, N-nitroso compounds, phenols, cresols, indoles, and $H_2$, rendering them unavailable as neurotransmitter precursors [61]. In particular, Trp degradation produces indole and indole-derived molecules that can modulate intestinal functions (permeability, endocrine and immune systems). Bacteria such as *Bacteroides, Lactobacillus, Clostridium, Peptostreptococcus, Fusobacterium, Porphyromonas*, and *Propionibacterium* are indole producers. *Bacteroides* and *Lactobacillus* were more abundant in HM-fed piglets, which could influence indole metabolism as reported [54]. Therefore, further investigations focusing on the amino acid-derived products of microbiota fermentation would be useful.

Plasma Trp levels were significantly lower in HM-fed than in IF-fed piglets, as previously observed [47]. In our study, the Trp:LNAAs ratio (Large Neutral AAs (LNAAs): His, Ile, Leu, Met, Phe, Tyr and Val [49,62]) was significantly higher ($P$=.003, data not shown) in the HM-fed piglets (1:31) than in IF-fed piglets (1:33), as previously observed [59]. This high ratio suggests that more Trp can enter the brain from HM plasma, because Trp and LNAAs compete for the same transporter, the LAT1, across the blood-brain barrier [55]. Accordingly, brain Trp levels were higher in the hippocampus of HM-fed piglets compared to IF-fed piglets. The higher, although nonsignificant, level of 3-HK, a kynurenine metabolite, in the hippocampus of HM-fed piglets than in that of IF-fed piglets may originated from the peripheral pool, as approximately 60% of kynurenine can cross the blood-brain barrier to participate in the synthesis of neuroactive metabolites in the central nervous system [63]. The 3-HK can lead to the production of the neuroprotective kynurenic acid (not analyzed in the present study) in astrocytes. Interestingly, serotonin levels in the brain regions were not diet dependent, except for the higher level of 5-HIAA, the primary metabolite of serotonin, in the brainstem of HM-fed piglets. The elevated level of 5-HIAA in the HM brainstem and 3-HK in the HM hippocampus may be related to the more abundant *Bifidobacterium* in HM-fed piglets, as reported in the brain of rats after oral administration of a *Bifidobacterium* strain [19,60]. Overall, these data demonstrate that HM feeding resulted in a preferential metabolism of Trp into the intestinal kynurenine pathway, leading to different levels of Trp, kynurenine and their derived metabolites, as well as different expression of genes involved in Trp metabolism in the colon, plasma and brain (hippocampus and brainstem) regions, all of which were correlated with some colonic bacterial abundances.

### *4.3. Plasma AA concentration differences are reflected in brain polyamine and neurotransmitter concentrations*

The levels of polyamines were lower in the colonic digesta (spermidine) and higher in plasma (putrescine) and several brain regions (NAP, putrescine, spermidine and spermine in the hippocampus; NAP and putrescine in the brainstem; putrescine in the hypothalamus and prefrontal cortex) in HM-fed than IF-fed piglets. The major source of intestinal polyamines stems from food intake and colonic microbiota production. No difference in polyamine levels was observed between HM and IF diets (Supplementary Table 3). Therefore, dietary modification of colonic bacteria could explain the differences in spermidine levels in colonic digesta and plasma putrescine levels after absorption through the intestinal mucosa and transfer to the bloodstream. Bacteria from Firmicutes and Proteobacteria phyla may be involved in such modulation of polyamine production, as suggested by significant correlations between polyamine levels and bacterial genera abundances in the colon. Conversely, since the brain cannot take up polyamines from the blood, polyamine levels in brain regions result from changes in the local synthesis of polyamines [64]. The higher levels of hippocampal putrescine, spermidine and spermine in HM-fed piglets may be related to higher levels of ornithine, as ornithine catabolism leads to polyamine production [65]. Met, *via* the generation of S-adenosylmethionine, also participates in the synthesis of spermidine and spermine. The elevated content of agmatine, a precursor of putrescine, in the HM hippocampus as well as in the HM brainstem and prefrontal cortex, may have enhanced the production of polyamines in these brain regions of HM-fed piglets. In the hippocampus, spermidine levels were positively correlated with genes involved in barrier function and involved in neurosynaptogenesis, suggesting a functional role for polyamines, particularly in the hippocampus. Finally, putrescine is a brain precursor of GABA [66]. Therefore, the higher hippocampal level of putrescine may partly explain the higher level of the neurotransmitter GABA in the HM-fed hippocampus.

The levels of Glu and Ile were higher in the hippocampus of HM-fed than IF-fed piglets, where Glu and Ile were positively correlated with some genes involved in barrier function, neurotransmitters and neurosynaptogenesis. These correlations emphasize the importance of Glu (neurotransmitter) and Gln (neurotransmitter precursor) in the brain, as previously highlighted [67], and suggest a higher hippocampal activity with HM feeding compared to IF feeding.

A limitation of our study was the sample size ($n$=9/group), which may limit the statistical power of the study. However, different strategies, including individual statistical tests and an integrative multifactorial analysis, result in highlighting potential effects of diet on gut and brain nitrogenous metabolomes. Another limitation is the targeted metabolomic approach, especially without considering bacterial metabolites.

## 5. Conclusion

This study highlighted that the infant diet, HM *vs*. IF, led to specific metabolome profiles, particularly in plasma and hippocampus. HM feeding resulted in a preferential metabolism of Trp into the intestinal kynurenine pathway, as illustrated by different levels of Trp-derived metabolites and gene expression in the colon, which may be partly explained by microbial changes. Brain levels of Trp and Trp-derived metabolites were higher mainly in the hippocampus of HM-fed piglets compared to IF-fed piglets, whereas sero-

tonin levels in the brain regions did not appear to be diet dependent. In addition, differences in brain AA levels resulted in different brain polyamine and neurotransmitter levels. This study also confirmed the specific metabolism occurring in each brain region, probably related to their specific functionality and nutrient needs.

It is interesting to note that the dietary (HM *vs.* IF) differences in gut microbiota can explain numerous differences in organ metabolomes. Further investigations are needed to pursue the optimization of IF through a better understanding of the health benefits of HM in terms of intestinal maturation, Trp metabolism and neurotransmitters release, and the HM components involved in such beneficial effects.

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## Data availability

The MFA and correlation datasets presented in this study can be found in an online repository. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.57745/ZVWTVX. Other data described in this article will be available upon request from the authors.

## Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## CRediT authorship contribution statement

**Elise Charton:** Writing – original draft, Methodology, Formal analysis, Visualization, Investigation. **Karl Fraser:** Writing – review & editing, Investigation, Funding acquisition, Methodology. **Paul J. Moughan:** Methodology, Funding acquisition, Writing – review & editing, Conceptualization. **Carlos A. Montoya:** Funding acquisition, Writing – review & editing, Conceptualization, Methodology. **Milson Francis:** Investigation, Methodology, Writing – review & editing. **Amandine Bellanger:** Resources, Writing – review & editing. **Nicole C. Roy:** Writing – review & editing, Conceptualization, Funding acquisition. **Didier Dupont:** Writing – review & editing, Conceptualization, Funding acquisition. **Amélie Deglaire:** Supervision, Visualization, Methodology, Conceptualization, Writing – review & editing, Funding acquisition. **Isabelle Le Huërou-Luron:** Methodology, Writing – review & editing, Visualization, Supervision, Funding acquisition, Conceptualization.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2025.110015.

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