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Authors: C. Watkins, K. Murphy, S. Yen, I. Carafa, C.A. O'Shea, E.A. Vercoe, R.P. Ross, C. Stanton, C.A. Ryan

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Effects of Therapeutic Hypothermia on the Gut Microbiota and Metabolome of Infants suffering Hypoxic-Ischemic Encephalopathy at birth

C Watkins[a,e], K Murphy[a,e], S Yen[f], I Carafa[g], C A O' Shea[d], E A Vercoe[f], R P Ross, C Stanton[a,b], C A Ryan[c,d].

Affiliations: [a]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland, [b]APC Microbiome Institute, UCC, [c] Infant Centre and Department of Paediatric and Child Health, University College Cork and [d]Department of Neonatology, Cork University Maternity Hospital, [e]Department of Microbiology, University College Cork, [f] Department of Molecular and Cellular Biology, University of Guelph, Ontario, Canada

Corresponding author: C.A. Ryan, Paediatrics & Child Health, Department of Neonatology, Cork, University Maternity Hospital, Wilton, Cork. T: +353-21-4920525.

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Introduction

The intrauterine environment impacts on foetal growth and development. Any interference on the interactions among foetus, placenta and mother is closely linked to the risk of developing long-term neuronal disorders, metabolic and cardiovascular diseases in postnatal life (Li et al., 2012; Warner and Ozanne, 2010).

Hypoxia, a condition in which the body experiences a loss of adequate oxygen, is one of the foetal stressing events capable of altering the brain development process and function, causing neurobehavioral, neuropsychological and neuropsychiatric disorders (Li et al., 2012). Traumatic birth, abruption of the placenta, umbilical cord compression, or failure of the neonate to successfully begin breathing are recognised as risk factors of the perinatal hypoxia-ischemia, which lead to Hypoxic Ischemic Encephalopathy (HIE) in 1 to 3 per 1000 full term births (Graham et al., 2008; Logitharajah et al., 2009; McGuire, 2007). It has been reported that roughly 20-25% of affected newborns die during the postnatal period whilst 25% of survivors with moderate HIE and 100% of survivors with severe HIE develop permanent neurologic injuries, including cerebral palsy, epilepsy, visual motor dysfunction

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and mental retardation (Douglas-Escobar and Weiss, 2015; Murray et al., 2016). When global perinatal hypoxia-ischemia occurs, dysfunctions can occur in multiple organs, including the gut (Nikiforou et al., 2016). Feeding intolerance, altered intestinal motility and necrotizing enterocolitis have been reported among the detrimental clinical effects affecting the gut of infants with perinatal hypoxia-ischemia (Berseth and McCoy, 1992; Fox and Godavitarne, 2012; Neu and Walker, 2011). Hypothermia is the most studied and optimized therapeutic method (Akula et al., 2015; Edwards et al., 2010; Eicher et al., 2005; Gunn and Thoresen, 2015; Gluckman et al., 2005; Schierholz, 2014; Thoresen et al., 2013) and has been incorporated in the Guidelines of International Consensus on Cardiopolmunary Resuscitation and Emergency Cardiovascular Care Science and in the recommendations of the American Heart Association in 2010 (Kattwinkel et al., 2010; Perlman et al., 2010). The hypothermic treatment involves reducing the body temperature of the infant within the first 6 hours (ideally within 3 hours) after ischemia in order to reduce the chance of detrimental effects, limiting cerebral damages, reducing the consumption of brain energy and reducing consumption of energy in the brain (Jacobs et al., 2013; Thoresen et al., 2013). The esophageal temperature is reduced to either 34.5 ± 0.5°C if the head is cooled, or 33.5 ± 0.5°C if the whole-body is cooled, and treatment is continued for 48–72 h (Jacobs et al., 2013).

The gut microbiota can be considered a dynamic organ of the human body, which continuously changes in composition and positively influences immunological and metabolic pathways, impacting the host health and wellness. Advances in research showed that the gut microbiota, as part of the gut-brain axis, can impact on host physiology and Central Nervous System performance including mood, cognition, other complex behaviours, and brain development (Forsythe et al, 2012; Stilling et al., 2014). At birth, development of the gastrointestinal microbiota in infants shifts towards a more adult-like status at two to three years of life (Costello et al., 2012; Relman, 2012). Many factors, e. g., gestational age, mode of delivery (spontaneous vaginal delivery or caesarean section), diet (breast milk or formula milk), sanitation, and antibiotic treatment (Adlerberth and Wold, 2009; Marques et al., 2010; Hill et al., 2017) influence the colonization process following delivery. Any dysregulation or alteration during this assembly process could contribute to adverse developmental programming and lifelong health outcomes. Both HIE and hypothermic therapeutic total body cooling (TBC) treatment represents disruptive events. The inflammatory process occurring during the first 24-48 hours post HI (Bona et al., 1999) and the low body temperature of

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infants exposed to TBC might influence the development and evolution of the gut microbiota, and its biological function. To our knowledge, the relation between hypothermia, biodiversity and metabolic function of the gut microbiota in infants who suffered perinatal HIE is unknown. The aim of this study was to investigate the impact of neuroprotection-induced hypothermia following birth on the evolution of gut microbiota in developing infants, and to describe the metabolomics profile of faecal samples in a group of full term infants with perinatal HIE.

METHODS

Patients and ethical approval

This was an observational study associated with infants who had undergone TBC in the Neonatal Unit at Cork University Maternity Hospital at birth. Infants who have undergone TBC due to HIE were recruited at birth and faecal samples were collected at 2 years of age (n=9). Faecal samples collected from infants in the INFANTMET study at 2 years were used as a control group for this study (n=8) (Hill et el., 2017). Once obtained, faecal samples were stored at -20 ˚C. Ethical approval was obtained from the Cork University Hospitals Research Ethics Committee and informed consent was obtained from the parents of all subjects. Infant demographics including apgar scores, mode of birth, gestational age, antibiotic administration, feeding regimes and severity of HIE diagnosis (Dx) were recorded (table 1).

Microbial DNA extraction and amplification

Microbial DNA was extracted from 0.2 g faecal samples using the Repeat Bead Beating (RBB) method described by Yu and Morrison, with some modifications (see supplementary material) (Morrison et al., 2004). The V3-V4 regions of the 16S rRNA gene were amplified and adapter sequences chosen according to the 16S metagenomic sequencing library protocol for the Illumina MiSeq using the following 16S primer pair F – (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and R- (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (Klindworth et al., 2013).

Bioinformatic Analysis

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300 bp paired-end reads were assembled using FLASH with parameters of a minimum overlap of 20 bp and a maximum overlap of 120 bp (Magoč et al., 2011). The QIIME suite of tools, v1.8.0, was used for further processing of paired-end reads, including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds (Caporaso et al., 2010). Denoising, chimera detection and operational taxonomic unit (OTU) grouping at 97% similarity were performed using USEARCH v7 (Edgar et al., 2010). OTU sequences were aligned using PyNAST and the SILVA SSURef database release 119 was used to determine taxonomy (Quast et al., 2013). Alpha and beta diversities were calculated using QIIME. Principal coordinate analysis (PCoA) plots were used to visualise differences in beta diversity based on UniFrac distances using EMPeror v0.9.3-dev (Vázquez-Baeza et al., 2013).

^{1}H NMR-Based Metabolomics

Fecal water samples were subjected to one dimensional proton nuclear magnetic resonance (^{1}H NMR) spectroscopy by preparing a mixture of 90% (v/v) sample and 10% (v/v) internal standard, which was used as a chemical shape indicator during metabolite quantification. The internal standard used was 5 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) in 99.9% D_2O and 0.2% (w/v) NaN_3. The sample mixture was transferred into 3 mm NMR tubes (Wilmad LabGlass, NJ, USA), and inserted into a Bruker Avance III 600.00 MHz spectrometer, equipped with a cryogenically-cooled triple resonance probe (TCI 600). Samples were scanned using a METNOESY pulse sequence, which is the first increment of a 1D-NOESY (1 s relaxation delay, 0.1 s mixing time and 4 s acquisition time). NMR spectra were processed using Chenomx NMR Suite 8.0 (Chenomx Inc., AB, Canada), which allowed for manual phase and baseline corrections. Metabolite assignment in this program was performed by matching compound signatures in the software database to peaks in the sample spectra. Concentrations were determined using the known DSS concentrations as a reference. Sample pH measurements were obtained after spectrum acquisition using Whatman™ pH indicator dip strips.

Statistical analysis

'R' statistical software package (version 3.2.4) was used for statistical analysis and a number of software packages were installed including vegan, ape, car, ggplot, rgl and ade4. Statistical significance in alpha diversity measures was assessed for Chao1, Shannon and Simpson diversity indices and observed species. Alpha diversity measures were calculated by using the

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phylogenetic diversity and observed species present in the dataset. Species richness, as measured by Chao 1 diversity index, can be described as a quantification measurement, calculating the number of different species present in a community, omitting the abundance of each species. In contrast, the Shannon and Simpson diversity indices were used to determine species diversity and eveness of the microbial dataset, taking into account the relative abundance of each species present in a community. To assess beta-diversity, bray-curtis distance matrices were generated on 'R' using the adonis function. Beta-diversity, defined as "the extent of change in community composition, or degree of community differentiation" (Whittaker, R.H. 1960), was visualized using PCoA. A non-parametric Kruskal-wallis test was used to determine statistical significance in the relative abundance of microbial taxa between the control and HIE treatment group. Statistical significance was accepted at $p<0.05$, with adjusted measures. A heatmap was also generated using Euclidean distance matrices on 'R' to compare microbial composition between groups. Clustering was performed with the hclust function in the R package gplots. Statistical analysis of 1H-NMR data was carried out using MetaboAnalyst 3.0 (Xia et al., 2016). Concentrations of metabolic compounds were normalised using a log transformation before performing statistical analysis. To determine whether the microbial dataset correlated with the metabolic data, co-inertia analysis was carried out using the ade4 package in 'R', using the Monte-Carlo test to generate an RV coefficient.

RESULTS

16S Microbial Composition

Diversity measures

During the course of this study a total of 17 faecal samples were collected from infants at 2 years of age and subjected to 16S rRNA analysis. Multiple alpha diversity measures were calculated (see supplementary material figure1). Phylotype richness was measured using the Chao 1 diversity index, while both species richness and evenness of the microbial dataset were measured using the Shannon and Simpson diversity indices. No significant differences were found using the Chao 1 diversity index, however the Shannon and Simpson diversity indices were found to be significantly higher in the control group ($p = 0.038$), compared to the HIE treated group. To assess whether there was any significant difference in beta-diversity between the two groups, a bray-curtis distance matrix was calculated and no significant difference was found ($Pr(>F) = 0.122$, see supplementary figure 2).

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Relative abundance of microbial taxa

The relative abundance of phyla present in both groups was determined from the rarefied dataset and compared for statistical significance (see figure1, 2 & 3, supplementary table 1). At phylum level, Firmicutes was found to be the dominant phylum in both groups, however at family level the abundance of *Lachnospiriaceae* was found to be significantly higher in the HIE treated group (52.22%), compared to the control group (36.38%) (p =0.0172). At genus level this result corresponded with a significantly higher abundance of *Lachnospiriaceae_Incertae.Sedis* in the HIE treated group (11.73%), compared to the control (6.98%) (p = 0.035) Indeed, Bacteroidetes was found to be significantly higher in the control group (8.65%), compared to the HIE treated group (2.30%) (p= 0.004), corresponding with significantly higher levels of *Bacteroides* at genus level in the control group (6.48%), compared to the HIE treated group (0.86%) (p = 0.002). Indeed, at genus level, high inter-individual variation was observed between both groups. In the control group, *Bifidobacterium* and *Blautia* were present at an average relative abundance of 15.07% and 9.16% respectively, however the relative abundance of these genera were found to be inverted in the HIE treated group with an average relative abundance of *Bifidobacterium* at 9.16% and *Blautia* at 15.49%. *Lachnospira* was also present in both the control and HIE treated group at an average relative abundance of 10.09% and 15.53% respectively. Interestingly, for two infants in the HIE treated group, *Akkermansia* was found to be quite high, with a relative abundance of 20.82% and 27.15% (see figure 3). Hierarchical clustering of samples, based on their relative microbial compositions, indicated that there were differences between the groups (see figure 4).

Metabolomic Data

Multivariate analysis was performed after sample normalisation using both principal coordinate analysis (PCA) and the partial least squared discriminative analysis (PLS-DA) in MetaboAnalyst3.0. PCA analysis revealed no clear separation between the control and HIE treated group in either 2D or 3D scores plots (see supplementary figure 4A and 4B). Subsequent PLS-DA was performed and found an initial separation between the two groups based on observations in the 2D and 3D scores plots (see supplementary figure 5A and 5B), however permutational analysis revealed no significance in separation distance after 100 permutations (p= 0.786 (786/1000). A PLS-DA variable importance measure was calculated using the weighted sum of the PLS-regression coefficients for the top 15 features (see

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supplementary figure 5C). Univariate analysis was also performed using a simple t-test ($p < 0.05$) and found no significant features between the control group, compared to the HIE treated group. Finally, a heatmap generated using Euclidean distance matrices on MetaboAnalyst3.0 indicated that there were no differences between the control and HIE treated group based on metabolic profile. This hierarchal clustering of samples highlighted high amounts of acetate across all samples in both groups, as well as low amounts of p-cresol, choline and formate (see figure 5).

Co-inertia analysis between 16S compositional sequencing data and 1NMR metabolomics data found no correlation between the two sample types (see supplementary figure 3, $p = 0.13$, $Rv = 0.525$)

Discussion

Microbial diversity and associated metabolic profiles have been extensively studied to investigate shifts or alterations in the gastrointestinal tract (GIT). For this study a total of 17 faecal samples were examined in children at 2 years of age to answer one question; would TBC treatment in patients with HIE at birth have an effect on the gut microbiota and faecal metabolic profile in later life? Indeed it has been suggested that asphyxia and hypoxic-ischemic injury may be the cause in some particular cases of necrotising entercolitis (NEC) (Young et al., 2011) (Torrazza and Neu, 2013). Thus, it is reasonable to suggest that this type of treatment at birth could impact the development of the gut microbiome.

Findings from this study suggest that TBC in treating HIE at birth had no significant effect on the gut microbial composition or metabolic profile of infants at 2 years of age. High inter-individual variation was observed between the control and HIE treated group, however analysis of microbial diversity found a significant decrease in the alpha diversity of our HIE treated group, suggesting that TBC may affect microbial richness in later life. In addition, a significant decrease in the relative abundance of *Bacteroides* was found in our HIE treated group, with an increased relative abundance of *Lachnospiriaceae*. This decreased abundance in *Bacteroides* may indeed have an impact on health outcomes in later life, with previous studies suggesting that a decreased abundance in Bacteroidetes is associated with an increased risk of developing obesity in later life (Kalliomäki et al., 2008) (Ley et al., 2005). Although differences in beta-diversity were non-significant, hierarchical clustering of samples, based on their relative microbial compositions, indicated that there was separation between the groups (see figure 4).

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Metabolic profiling of faecal samples detected acetate as the most abundant compound present in samples across both groups (see figure 5). Indeed, concentrations of faecal acetate have previously been reported to be higher in breast fed infants compared to formula and mixed-fed infants (Kleesen et al., 1995) (Siigur et al., 1993). Production of acetate has also been found to be anti-inflammatory in the regulation of colonic T-reg cells (Smith et al., 2013), whilst absorption of acetate in the colon has also been found to increase cholesterol synthesis (Wong et al., 2006). Overall, high inter-individual variation was observed in the metabolic profile in both the control and HIE treated group. Metabolic compounds which correlated with the increased abundance of acetate included pyruvate, glucose, 4-aminobutyrate and 5-aminopentanoate (see supplementary figure 6). Microbial metabolism of essential amino acid substrates has been found to increase metabolic by-products, including short chain fatty acids (SCFAs), acetate and butyrate, and branched chain fatty acids (BCFAs), isobutyrate and isovalerate. Moreover, production of these particular microbial by-products has been found to improve colonocyte health and mucosal immunity (Blachier et al., 2007).

Overall, results from this study suggest that there is no negative impact to the gut microbial development of infants subjected to TBC at birth, compared to a healthy full term infant group at 2 years of life. To our knowledge this is the first study to report the gut microbial composition and metabolic profile in infants exposed to this type TBC treatment at birth.

A limitation to this study included the small sample size in each group, in addition to the time period in which the samples were collected. Faecal samples were collected at 2 years of life and thus a number of confounding factors must be considered. Firstly diet is a very important factor which can affect the faecal metabolome and microbiota profile after birth, in particular the effect of human breast milk (HBM) during the first year of life. A review by Fanos et al., 2015 concluded that further studies are necessary to evaluate the HBM metabolome in a variety of infant cohorts, with particular emphasis on breast milk collected from mothers' of full term and preterm infants. Indeed the metabolome of preterm HBM has been found to have increased concentrations of lactose in comparison to full term HBM (Marincola et al., 2012). In addition, HBM can be classified as 'Secretor' or non-Secretor' milk due to the presence or absence of fucosyltransferase genes (FUT2 or FUT3), which in turn effect the structure of milk glycans and alter the bifidobacterial communities present in the gut (Lewis et al., 2015). In terms of metabolic profile, a recent study found that breast feeding strongly influenced the faecal metabolome of infants where a significantly increased abundance of

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acetate was found in exclusively breast fed infants, in comparison to a non-breast fed infant group; independent of delivery mode, intrapartum antibiotics, age and sex (Bridgman et al., 2017).

Thus, limitations to this study include the lack of metabolomic information on HBM supplied to the infants, in addition to the fecal metabolic profile during the first year of life. However, a standardized approach was followed during recruitment of the study and each infant received HBM from mothers whom delivered full term at birth. Previous studies have correlated the faecal metabolic and microbial profiles of healthy infants' overtime and have provided findings which provide insight to the current dataset in this study. For example, a significantly increased abundance of acetic acid and bifidobacteria was found in one breast fed infant cohort (Liu et al., 2016), and a study by Del Chierico et al., 2015 found a low abundance of acetate and butyrate which correlated with an increased abundance of Proteobacteria in a small cohort of breast fed and formula fed infants during the first 30 days of life (Del Chierico et al., 2015). Moreover, the INFANTMET study, from which the control group for this study were recruited, has described the microbiota profile in the full term infant cohort, of both caesarean delivered and spontaneous vaginally delivered infants, to be dominated by similar levels of *Bifidobacterium* (average of ~52%) and *Bacteroides* (average of ~12%) at 6 months of age (Hill et al., 2017), however at 2 year of age the abundance of these genera decrease to 15% and 6% respectively, in this small subset of infants.

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Captions for Figures

Figure 1. Percentage relative abundance of microbial taxa at phylum level.

Figure 2. Percentage relative abundance of microbial taxa at family level.

Figure 3. Percentage relative abundance of microbial taxa at genus level.

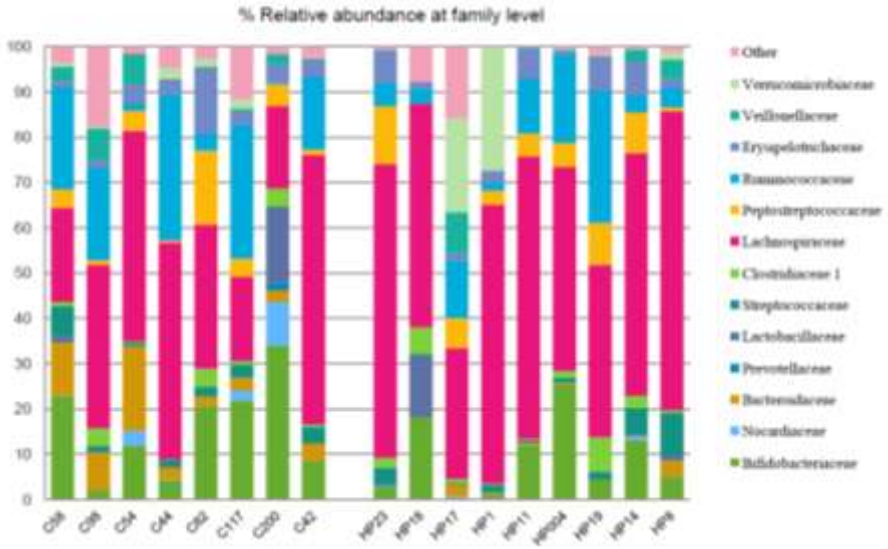
Figure 4. Hierarchically clustered heat map showing correlations between the relative microbial compositions in each group (control = HPC, HIE treatment group = HP). Clustering was performed with the hclust function in the R package gplots. The color of each tile of the heat map indicates the strength of the correlation for a given genera. (blue = 2, light blue = 1, orange = 0, green = -1, yellow = -2)

Figure 5. Heatmap of 1H-NMR faecal metabolomic data using Euclidean distance matrices on MetaboAnalyst 3.0

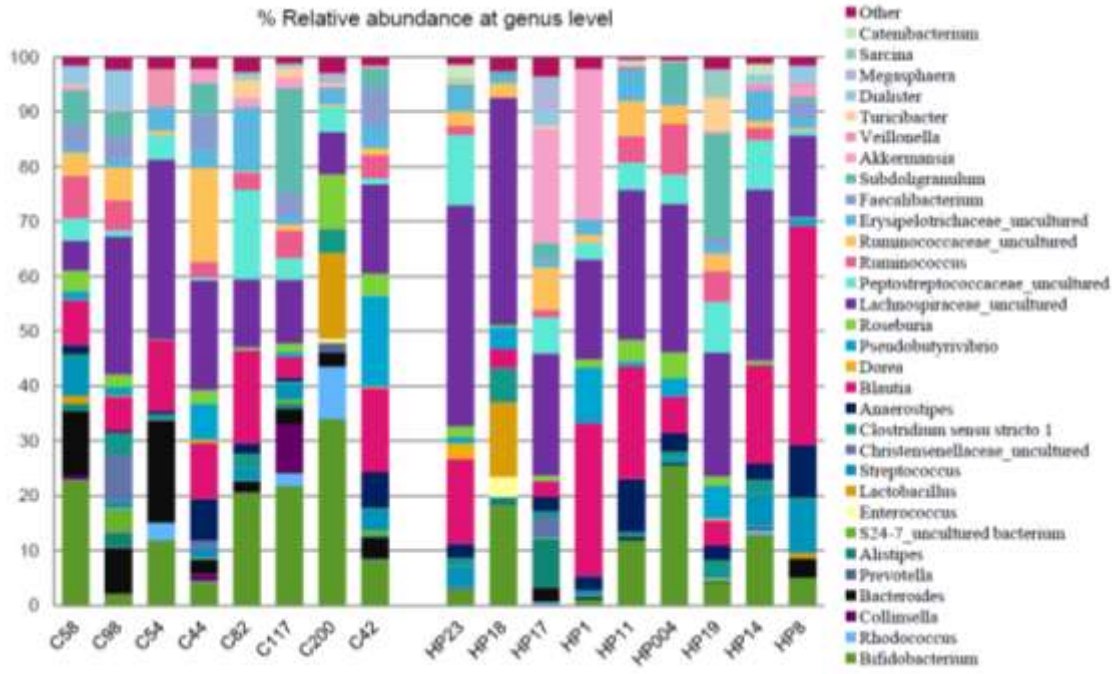
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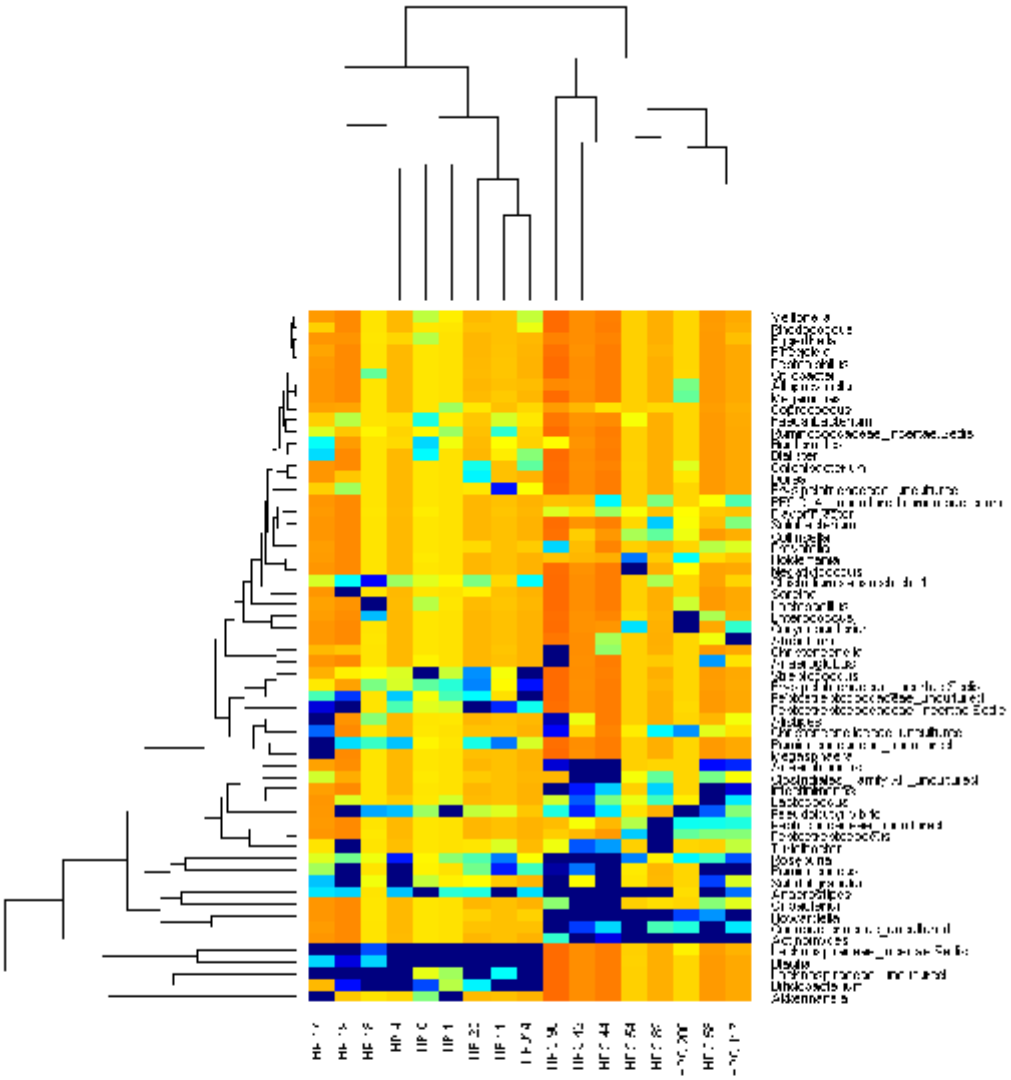
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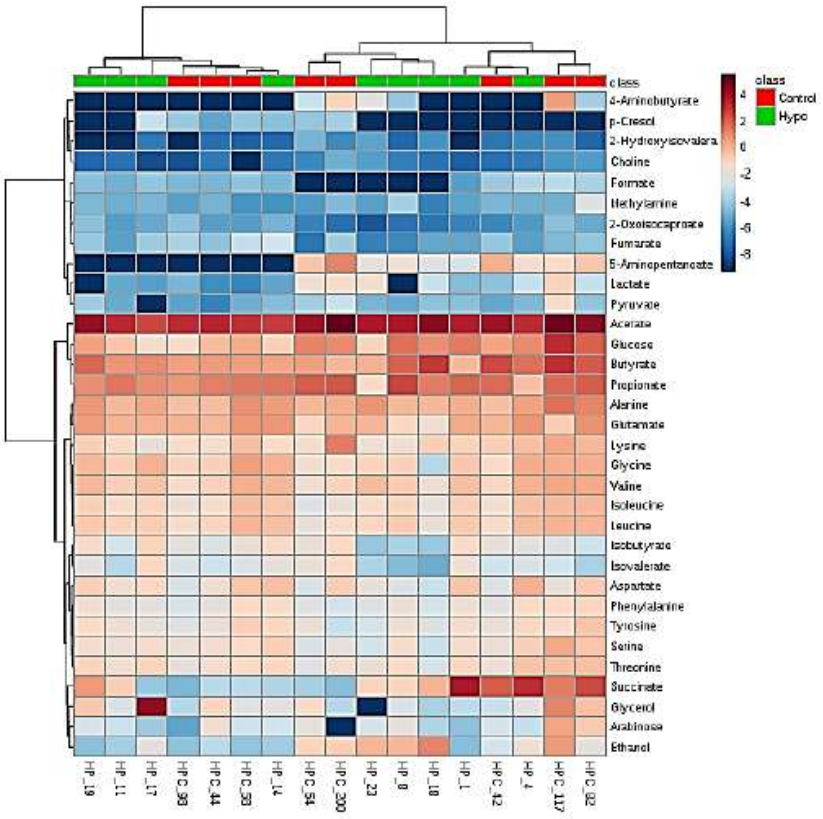
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	Subject number	Apgar (1min)	Apgar (5min)	Gestation (weeks)	Delivery of Mode	Mode of feeding (<4months)	Antibiotics at birth	Dx
HIE Patients	HP 1	2	3	41+3	Vac - Vaginal	Formula	yes	Hypotraemia
	HP 4	3	4	36+2	breech - Vaginal	Breast Milk	yes	Left temporal extradural haematoma associated overlying cephalhaematoma
	HP 8	1	3	38	SVD	Breast Milk	yes	Severe perinatal asphyxia
	HP 11	5	7	40+6	Vac-Vaginal	Breast Milk	yes	HIE Grade 2 - Moderate Neonatal Encephalopathy
	HP 17	3	6	41+3	Vac-Vaginal	Breast Milk		HIE Grade 2 - Moderate
	HP 18	4	5	41+6	CS	Breast Milk	yes	HIE Grade 2 - Moderate
	HP 19	6	8	40+1	Vac-Vaginal	Breast Milk	yes	HIE Grade 2 - Moderate
	HP 23	0	4	39+4	CS	Breast Milk	yes	
	HP 14	1	5	39+5	CS	Breast Milk + Formula	yes	HIE Grade 2 - Moderate
INFANTMET Control	HPC 117	9	10	39+2	CS	Breast Milk	yes	
	HPC 54	9	9	39+1	SVD	Breast Milk	no	
	HPC 98	9	10	39+5	CS	Breast Milk	yes	

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	HPC 200	7	9		SVD	Breast Milk	no	
	HPC 44	9	9	40+2	SVD	Breast Milk	yes	
	HPC 42	9	10	38	SVD	Breast Milk	yes	
	HPC 82	9	10	39+1	CS - Emergency	Breast Milk	unknown	
	HPC 58	9	9	37+2	SVD	Breast Milk	yes	

Table 1. Patient information for both groups (**HP** = hypothermia treated, **HPC** = control).