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Fetal cardiac growth is associated with in utero gut colonization

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Abstract *Background and aims:* Intra-uterine metabolic environment predicts newborns' cardiac morphology, metabolism and future health. In adults, gut microbiota composition relates to altered cardiac structure and metabolism. We investigated the relationship between gut microbiota colonization and fetal cardiac growth.
Methods and results: Bacterial composition in meconium samples of 26 healthy, full-term newborns was assessed by 16S rDNA gene sequencing. Its relationship with birth echocardiographic parameters, and the interaction with cord blood levels of inflammatory markers were investigated. Correlative and cluster analysis, linear discriminant analysis effect size and predictive functional analysis based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were applied. Fetal left ventricle growth was related to gut microbiota composition at birth. Specifically, left ventricle posterior wall thickness (LVPW) greater than 4 mm was associated with lower microbiota beta and alpha diversity, depletion (LDA score > 3) of several bacteria at each taxonomic level, including *Lactobacillales*, and enrichment (LDA score > 5) in *Enterobacteriales* and *Enterobacteriaceae*. The latter was significantly related to cord blood gamma-glutamyltransferase levels ($r = 0.58$, $p = 0.0057$). Functionally, a thicker LVPW was related to up-regulation of pathways involved in lipopolysaccharide biosynthesis (+50%, $p = 0.045$ in correlative analysis) and energy metabolism (+12%, $p = 0.028$), and down-regulation of pathways involved in xenobiotic biodegradation (−21 to −53%, $p = 0.0063$–0.039), PPAR signaling (−24%, $p = 0.021$) and cardiac muscle contraction (−100%, $p = 0.049$).
Conclusion: Fetal cardiac growth and gut colonization are associated. Greater neonatal LVPW thickness is related to lower diversity of the gut microbiota community, depletion of bacteria having anti-remodeling effects, and enrichment in bacteria functionally linked to inflammation.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSA, body surface area; CCA, canonical correspondence analysis; EDD, end-diastolic diameter; EDV, end-diastolic volume; ESD, end-systolic diameter; ESV, end-systolic volume; GGT, gamma-glutamyltransferase; IL-6, interleukine-6; IVSd, interventricular septum diameter; LDA, linear discriminant analysis; KEG, Kyoto Encyclopedia of Genes and Genomes; LPS, lipopolysaccharide; LVEF, left ventricle ejection fraction; LVPW, left ventricle posterior wall diameter; MCP-1, monocyte chemoattractan protein-1; OTU, operational taxonomic unit; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states; PPAR, peroxisome proliferator-activated receptor; TNF-alpha, tumor necrosis factor alpha.

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Introduction

According to the developmental origin of health and disease hypothesis, subclinical cardiovascular disease begins during fetal life [1]. In fact, fetal growth has been linked to left ventricular (LV) structure and risk of coronary artery disease in later life [2–4]. Moreover, LV hypertrophy is a strong predictor of cardiovascular morbidity in adulthood [5]. We recently showed [6] that maternal overweight during pregnancy predicts greater left ventricular thickness and mass in newborns, and that elevation in myocardial glucose uptake and suppression of glycolysis, Krebs cycle, electron transfer, and oxidative enzyme expression may underlie cardiac morphological changes.

Fetal life is important for gut microbial colonization, as bacteria have been detected in placenta [7–9], umbilical cord [10] and meconium [7,11,12], both in vaginally and C-section delivered newborns. There is growing interest, but still very limited knowledge, concerning the role of the gut microbiota in the pathogenesis of cardiovascular disease. Crawford et al. showed [13] reduced cardiac mass and increased myocardial glucose oxidation in germ-free compared to mice transplanted with microbiota from conventionally raised mice, in spite of no difference in cardiac function, providing a direct link between gut microbiota composition and cardiac metabolism and morphology. The lack of microbiota-dependent delivery of ketone bodies might underlie the increased glucose utilization in germ free compared to conventionalized mice [13]. In addition, changes in gut microbiota were found in a mouse model of myocardial infarction, and were implicated in the beneficial cardiovascular effects of exercise training [14]. In humans, altered abundances of specific bacterial taxa, including *Coriobacteriaceae*, *Erysipelotrichaceae*, *Ruminococcaceae*, *Blautia*, *Collinsella*, *Faecalibacterium and Enterobacteriaceae*, and reduced diversity have been found in the gut microbiota of patients with heart failure compared to healthy controls [15]. In both studies [14,15], the author postulated that chronic systemic inflammation associated with myocardial infarction and heart failure might compromise intestinal epithelial function, microbiome composition and its metabolic and immune effect, which in turn might exacerbate the primary inflammatory disease in a vicious cycle.

In the present study, we hypothesized that gut microbiota and cardiac growth during fetal life are associated. In the above cited population of newborns [6], we assessed bacterial composition in meconium samples by 16S rDNA sequencing, and explored the relationship between gut microbiota and echocardiography parameters at birth.

Methods

Study population

The study was conducted in 26 full-term newborns from the Pisa birth cohort [6]. Neonatal gestational age, body size, meconium, and cord blood samples (N = 21–24) were collected at birth, and cardiac parameters were measured within 2 days after birth by trans-thoracic echocardiography (GE Logic, 2.5–6.0 MHz transducer) as previously described [6]. Maternal anthropometrics were measured during pregnancy.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Massa and Carrara, and latest amendments by the Ethical Committee of the Area Vasta Nord-Ovest (CEAVNO), Pisa, Italy. Parents gave their written informed consent before inclusion.

Analysis of metabolic and inflammatory markers in cord blood

Cord blood levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT) were analyzed by routine enzymatic methods (Synchron CX9, Pro Beckman Coulter, Inc, Brea, CA, USA). Circulating levels of tumor necrosis factor alpha (TNF-alpha), interleukine-6 (IL-6), and monocyte chemoattractan protein-1 (MCP-1) were measured by Luminex® xMAP® technology (Milliplex map kit, EMD Millipore Corp., MA, USA). Procedures were carried out according to manufacturer's instruction.

Analysis of microbiota in meconium

Bacterial genomic DNA was extracted from meconium samples pre-treated with mutanolysin and lysozyme (Sigma–Aldrich, Saint Louis, MO) [16], by using the Master Pure Complete DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. The V3–V4 region of the 16S RNA gene was amplified by PCR (KAPA HiFi HotStart DNA Polymerase, Kapa Biosystem, Cape Town, South Africa) using the universal bacterial primers [17,18], according to 16S Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA). After quality and quantitative checks, libraries were sequenced on MiSeq platform (Illumina, San Diego, CA) in a 2 × 300 pb paired-end format. The raw sequence files generated underwent quality control analysis with FastQC.

Bioinformatics and statistical analysis

Quality assessment was performed by using Trimmomatic software (v0.36) [19], amplicon reconstruction was performed by using PEAR (v0.9.10) [20], and chimeric sequences and human contaminants were removed [21]. The remaining reads were clustered into OTUs at 97% identity using the open-reference OTU picking approach of the QIIME 1.9.1 package [22], and classified based on the Greengenes (v13_8) database.

Calypso software v8.20 was used, with total sum normalization of data for statistical analysis. Spearman's correlation analysis was used to explore univariate associations between continuous variables, and Adonis analysis (Bray–Curtis, Chao, Euclidean metrics) was applied to assess gut microbiota beta diversity based on

echocardiography parameters. Multivariate method canonical correspondence analysis (CCA) allowed testing complex associations between gut bacteria communities and cardiac parameters. The latter CCA analysis was also exploited to identify data driven cut-off in cardiac parameters that could be associated with clearly different microbial populations and have clinical relevance. Then, alpha-diversity indices Shannon, Chao1, Simpson, Fisher-alpha, richness and evenness metrics were analyzed between groups identified by CCA. Rank-test and linear discriminant analysis effect size (LEfSe), were used to identify unique biomarkers (LDA score > 3.0). Predictive functional analysis was performed using PICRUSt with Kyoto Encyclopedia of Genes and Genomes (KEGG) [23].

SPSS for MAC (version 22, Chicago, IL, USA) was used to assess data distribution, and parametric and non-parametric tests were applied as appropriate. FDR-correction was applied in multiple comparisons analyses. The threshold significance level was set at 0.05.

Results

Study population and gut microbiota composition

Newborns were 57.7% male, 73.1% were born by vaginal delivery, all were born at term. Birth body size and echocardiographic parameters were within the normal range, and are reported in Table 1, together with cord blood concentrations of circulating markers, and maternal pre/end-gravidic BMI. Gestational diabetes mellitus (GDM) was present in 6 (23.1%) mothers.

In the whole population, most abundant bacteria were *Firmicutes* (43.15%), *Proteobacteria* (35.00%), *Actinobacteria* (17.28%), *Bacteroidetes* (1.87%), and *Verrucomicrobia* (1.28%) at phylum level, *Clostridiales* (27.27%), *Enterobacteriales* (27.03%), *Bifidobacteriales* (14.77%), *Lactobacillales* (12.50%), *Xanthomonadales* (4.03%), *Bacillales* (2.33%), *Bacteriodales* (1.57%), *Actinomycetales* (1.46%) and *Verrucomicrobiales* (1.28%) at order level.

Table 1 Study population.

	N	Mean	Std. Deviation
Vaginal delivery, N (%)	19 (73.1)	–	–
Male gender, N (%)	17 (57.7)	–	–
Maternal GDM	6 (23.1%)		
Maternal pre-gravidic BMI (kg/m^2)	26	24.3	4.1
Maternal end-gravidic BMI (kg/m^2)	23	28.8	3.8
Gestational age, days	26	280.5	9.6
Birth height, cm	26	50.2	2.0
Birth weight, g	26	3384	360
Apgar 1 min	26	9.0	0.3
Apgar 5 min	26	9.7	0.5
Cardiac parameters			
LVEF% m-mode	25	72.6	8.4
LVEF% b-mode	26	70.3	7.1
EDD, mm	26	16.9	2.4
ESD, mm	26	10.5	2.1
EDV, ml	26	3.6	1.0
EDV/BSA, ml/m^2	26	16.8	5.2
ESV, ml	26	1.1	0.4
ESV/BSA, ml/m^2	26	5.3	2.2
IVSd, mm	26	4.1	0.7
LVPWd, mm	26	3.6	0.8
Mass g	26	9.1	2.5
Mass, g/m^2	26	43.4	10.7
E/A, m/sec	26	1.14	0.31
Stroke volume, ml	26	2.6	0.8
Cord blood circulating markers			
IL-6 (pg/ml)	23	18.4	30.5
TNF-alpha (pg/ml)	24	11.3	4.8
MCP-1 (pg/ml)	24	435.9	151.5
GGT (pg/ml)	21	127.3	113.2
AST (pg/ml)	22	66.9	101.1
ALT (pg/ml)	21	12.2	5.0

Abbreviations: GDM = gestational diabetes mellitus; LVEF = left ventricle ejection fraction; EDD = end-diastolic diameter; ESD = end-systolic diameter; EDV = end-diastolic volume; ESV = end-systolic volume; BSA = body surface area; IVSd = interventricular septum diameter in diastole; LVPWd = left ventricle posterior wall diameter; IL-6 = interleukine-6; TNF-alpha = tumor necrosis factor alpha; MCP-1 = monocyte chemoattractan protein-1; GGT = gamma-glutamyltransferase; AST = aspartate aminotransferase; ALT = alanine aminotransferase.

Microbiota and echocardiographic parameters

Several cardiac variables were significantly correlated with gut bacteria abundances in univariate analysis. However, only correlations with LVPWd survived FDR-correction (Fig. 1A), and LVPWd was the only cardiac parameters significantly and consistently (among metrics) associated with microbiota beta diversity (Supplementary Fig. 1A).

Multivariate CCA identified distinct bacteria community clusters at class ($p = 0.035$), order ($p = 0.009$), family ($p = 0.024$) and OTU ($p = 0.050$) levels only in relation to birth LVPWd, and not to other cardiac parameters. Specifically, based on the LVPWd continuous variable, CCA identified two separate clusters associated with LVPWd above ($n = 7$ newborns) or below ($n = 19$ newborns) the data driven cut-off of 4 mm (Fig. 1B). This indicates that LVPWd significantly ($p = 0.009$) explains the variations observed in the gut microbiota. Similar results were obtained by PCA ($p = 0.05$ family and order levels, $p = 0.035$ class level). Since this threshold has clinical relevance, representing the upper limit in the 95% confidence interval of age-matched population-based LVPW dimension [24], further analysis was performed to deepen its relationship with specific gut bacteria ad their functions.

Microbiota composition in newborns with thick or thin LVPW

Babies were stratified in those born with thin or thick LVPW (</>4 mm). Groups were similar for gestational age, birth anthropometric parameters, male gender prevalence and type of delivery. Maternal GDM was similarly distributed between groups, and not related to LVPWd. Conversely, higher end-gravidic BMI tended to

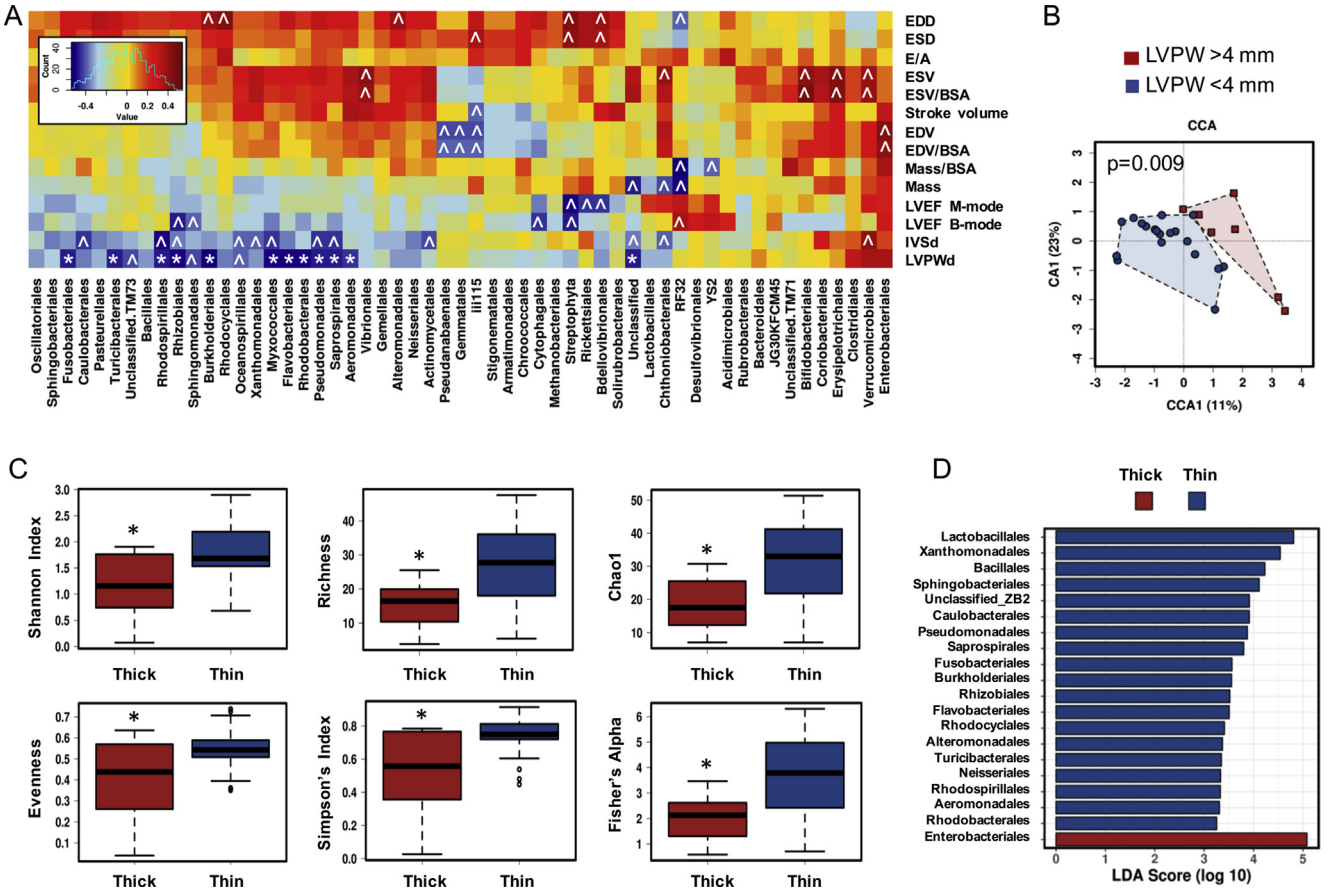

Figure 1 Relationship between echocardiographic parameters and gut bacteria. (A) The heatmap shows Spearman's correlative coefficients, highlighting a strong interaction between gut microbiota (at order level) and LV posterior wall diameter (LVPW), ^ $p < 0.05$ in univariate analysis, * $p < 0.02$ in univariate and $p < 0.1$ in FDR-corrected analyses. (B) Canonical correspondence analysis (CCA) shows significant clustering ($p = 0.009$) of microbiota community based on LV posterior wall diameter (LVPW) below or above a threshold value of 4 mm (data driven). (C) Alpha diversity calculated by several indices was significantly lower in thick compared to thin LVPW group. (D) Nineteen key bacteria (at order level) were identified in the thin LVPW group and only 1 in the thick LVPW group by LEfSE analysis (LDA > 3).

associate with thicker offspring birth LVPWd ($p = 0.077$), confirming published data [6]. Cord blood levels of inflammatory markers and hepatic enzymes were not different between groups, nor associated with LVPWd (Supplementary Table 1).

Instead, significant group differences were found in gut microbiota diversity, as newborns with thick LVPWd had lower alpha diversity at order (Fig. 1C) and other taxonomic levels (Supplementary Table 2).

LEfSE and Wilcoxon rank-test analyses showed that 19 order taxa, *Lactobacillales Xanthomonadales*, *Bacillales* and *Pseudomonadales* as the most relevant (Supplementary Fig. 1B), were significantly enriched in newborns with thin LVPWd, while only *Enterobacteriales* were enriched in those with thick LVPWd (Fig. 1D). A greater number of taxa was associated with thin compared to thick LVPW also at phylum (4 vs 0), class (9 vs 0), family (27 vs 1), genus (26 vs 0) and OTU (12 vs 2) levels. *Enterobacteriales* and *Enterobacteriaceae* were consistently elevated in the thick LVPW group, and positively correlated with cord GGT levels ($r = 0.58$, $p = 0.0057$).

The adjustment for maternal end-gravidic BMI (to assess its potential confounding effect) did not abolish group differences in *Enterobacteriales* and *Lactobacillales* abundances.

Functional alterations in microbiota of newborns with thick or thin LVPWd

KEGG analysis revealed 223 pathways associated with meconium microbiota. In newborns with thick LVPW, we found an up-regulation of energy metabolism (12%) and carbohydrate metabolism pathways, and a down-regulation of pathways involved in lipid and aminoacid metabolism, including linoleic acid and ketone bodies (−38%) metabolism, and valine, leucine and isoleucine degradation (−26%) (Fig. 2A). Interestingly, lipopolysaccharide biosynthesis proteins were positively correlated with LVPW thickness ($r = 0.40$, $p = 0.045$), and increased by 50% in the thick LVPW group (Fig. 2B). Pathways associated excretory system (+35%) and type II diabetes mellitus (+10%) were also up-regulated. In this same group several xenobiotic biodegradation pathways were down-regulated (between −21 and −53%), together with peroxisome proliferator-activate receptor (PPAR) signaling

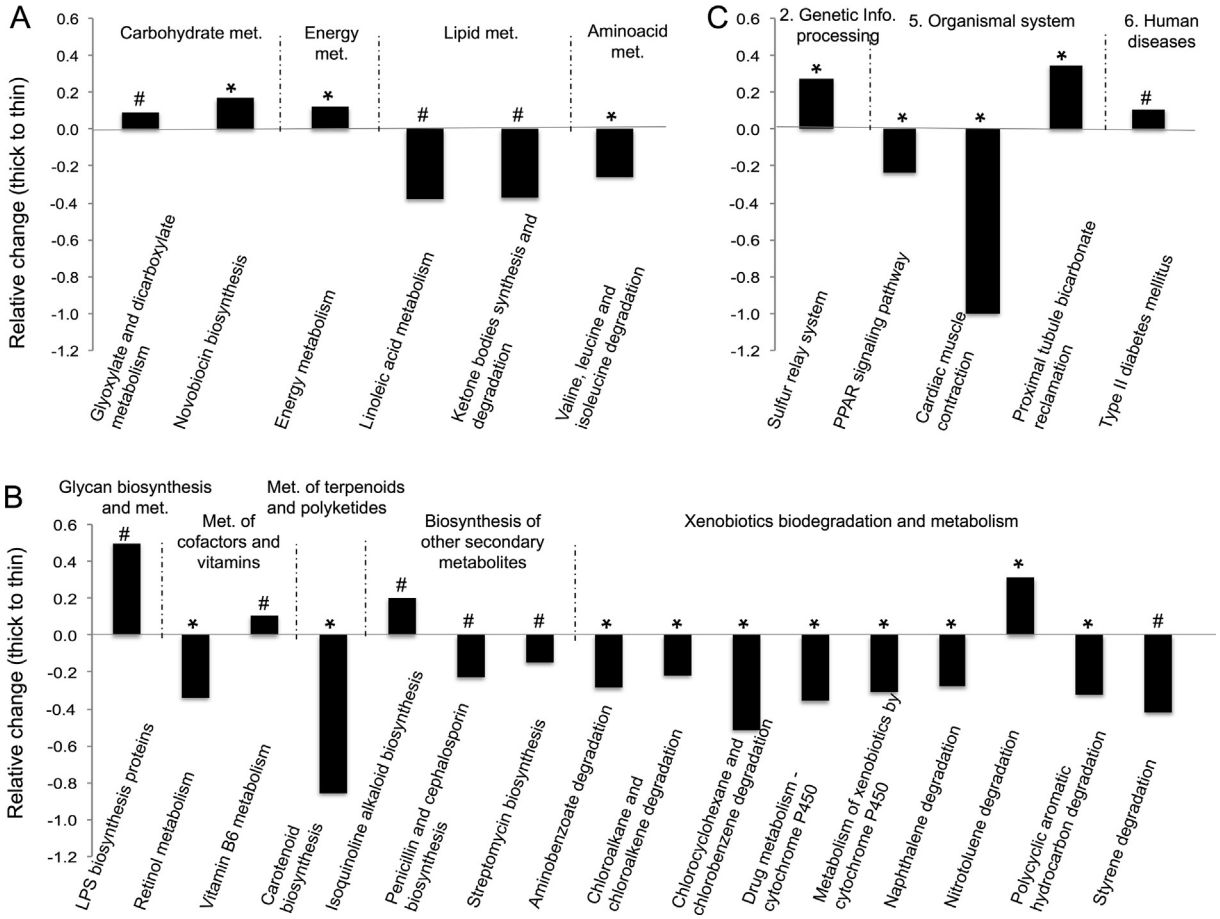

Figure 2 Predictive functional analysis. Predictive functional analysis using PICRUSt with Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed differentially regulated (relative change >10%) metabolic and disease-related pathways in newborns with thin or thick LVPW. Differentially regulated pathways belong to KEGG categories 1 = Metabolism (A–B, subcategories indicated in the figure); 2 = Genetic Information Processing, 5 = Organismal System, and 6 = Human Diseases (C); *p < 0.05 in rank test, #p < 0.1 in rank test and p < 0.05 in Spearman's correlation. Abbreviation: met. = metabolism.

(−24%) and cardiac muscle contraction (−100%; r = −0.61, p = 0.001 in correlative analysis) (Fig. 2C).

Discussion

We showed that gut colonization starts in utero, and is associated with LVPW thickness at birth. Specifically, newborns with thicker LVPW are characterized by reduced gut microbiota diversity, depletion of bacterial taxa functionally involved in the regulation lipid and protein metabolism and ketogenesis pathways, and enrichment in those involved in the synthesis of pro-inflammatory LPS, energy and carbohydrate metabolism. In particular, we identified depletion of *Lactobacillales* and enrichment in *Enterobacteriales*. We performed two types of main analysis. The first was based on correlations between continuous variables, which could robustly demonstrate the concept. The second was aimed at identifying a LVPWd cut-off that could be associated with clearly different microbial communities and metabolic pathways, to support clinical orientation.

The first finding of this study is that a diverse microbial community populates the gut of newborns. Our data are in line with recent evidence showing the occurrence of bacteria not only in meconium [7,11,12], but also in the placenta [7–9], amniotic fluid [7], and umbilical cord [10], in contrast with the sterile-womb paradigm. The origin of the intrauterine colonization is still unknown, but preclinical studies have shown that labeled bacterial administered orally to pregnant dams can be isolated in placenta and meconium samples [25], suggesting that maternal intestinal microbiota might be transported to the feto–placenta interface.

The second finding is that the newborn gut microbiota community is associated with fetal cardiac growth, in particular LVPW thickness. The metabolic and nutritional environment to which the fetus is exposed has impact on its risk to develop cardiovascular disease during adulthood [3,26]. We previously showed that maternal end-gravidic BMI predicts newborns' LVPW thickness and mass, as well as altered myocardium metabolism [6]. Moreover, there is growing evidence [27,28] that maternal health during pregnancy affects early gut colonization. We postulated that gut microbiota modulates cardiac growth during fetal development.

Reduced beta and alpha diversity of gut microbiota have been reported in preclinical models of heart disease [14]

and in patients with heart failure [15], where gut microbial beta diversity was significantly associated with echocardiographic parameters, including LVEF and LV-ESD [14]. Overall, the decrement, rather than overgrowth, of bacterial taxa underlies a complex remodeling of intestinal bacterial structure associated with heart failure. Our data show that this association starts in the earliest stage of life, when the microbiota is more tightly related to cardiac thickening than function. Consistently, the complete absence of bacteria in germ-free compared to control mice resulted in reduced cardiac mass, and no effect on cardiac function [13]. In germ-free mice, cardiac abnormalities were explained by metabolic abnormalities, including the lack of microbiota-dependent delivery of ketone bodies, and the compensatory up-regulation of myocardial glucose utilization during nutrient deprivation [13]. Consistently, in our thick LVPW group, we found up-regulation of pathways regulating carbohydrate and energy metabolism, and down-regulation of those involved in lipid and protein metabolism, including reduced PPAR signaling and ketogenesis, Overall, the current knowledge suggests that microbiota-dependent modifications of these metabolic pathways might be implicated in cardiac wall thickening.

Identification of specific bacteria involved is important to devise new strategies to promote cardiac health. In newborns with thick LVPW, we found depletion of Lactobacillaes and enrichment in Enterobacteriales. Gan at al [29]. showed an anti-remodeling effect (attenuation of LV hypertrophy) of the probiotic *Lactobacillus rhamnosus* GR-1 compared to placebo when administered to rats during 6 weeks of sustained coronary artery occlusion. Also, enrichment in *Enterobacteriaceae* was shown in patients with heart failure [15] and atherosclerotic disease [30]. In the latter, enrichment in genes required for synthesis of the O-antigen of lipopolysaccharides (LPS) was also reported, suggestive of a pro-inflammatory gut microbiota. Consistently, in our study LVPW thickness was associated with up-regulation of the LPS biosynthesis proteins pathway (functionally linked to *Enterobacteriaceae* enrichment), and down-regulation of the cardiac muscle contraction pathway. Moreover, *Entrobacteriaceae* abundance and cord levels of the pro-inflammatory enzyme GGT, which has been strongly related to cardiac conditions [31,32], were directly correlated. It is tempting to speculate that the pro-inflammatory profile induced by the gut microbiota promotes ventricular growth during fetal development, and modulates the risk of cardiac disease in later life. This remains to be proven in an ad hoc cause-effect design.

We defined thin or thick LVPW as below or above 4 mm, based on the (data driven) clustering of gut microbiota in CCA. Although defining a clinical threshold was not an endpoint of the present work, it is of note that 4 mm represents the upper limit in the 95% confidence interval of age-matched, population-based LVPW data [24].

In the present study, delivery mode was not used as covariate since vaginal and C-section delivery were similarly distributed between the two groups. Though the delivery mode has been reported to affect early gut microbiota [12,33], the most recent evidence indicates that this association occurs in subsequent transitional stool but not in meconium, which should instead reflect intestinal colonization before labor and birth [34,35].

To the best of our knowledge, this is the first study exploring the relationship between intestinal bacteria and cardiac development during fetal life in a well-characterized birth cohort. Limitations have to be acknowledged, including the small sample size and the inter-individual variability. Moreover, our results are descriptive, which only allows speculating on mechanisms and cause-effect links between gut bacteria and cardiac growth.

In conclusion, our data confirm that gut colonization starts in utero and provide first evidence of bacteria associated with cardiac development during fetal life. Our results provide the evidence base that is necessary to support ad hoc mechanistic designs, and open the perspective of microbiota-based modulation of cardiac health since fetal life.

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Conflicts of interest

The authors have no conflicts to disclose in relation to the current manuscript.

Authors’ contributions

MAG: coordination of sample and data collection, data analysis and interpretation, manuscript writing, LAA: echocardiography analysis and expertise in neonatal cardiology, RD and MP: bioinformatics analysis, ES: contribution to analysis, FR, PS and AW: bacterial DNA extraction and sequencing, PI: study conception and design, manuscript writing and project funding. All the authors have contributed to data interpretation, and have critically revised and approved the current version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.numecd.2018.10.005.

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