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ORIGINAL ARTICLE

Urinary metabolome of infants with colic treated with *Lactobacillus reuteri* DSM 17938: a pilot randomized trial

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ABSTRACT

BACKGROUND: *Lactobacillus reuteri* DSM 17938 is the only probiotic recommended for treatment of colicky infants, but its mechanism of action is not clear. The study aim was to examine urinary metabolomic fingerprint of colicky breast-fed infants before and after 1 month of orally administered *Lactobacillus reuteri* DSM 17938 or placebo.

METHODS: This randomized, blinded, placebo-controlled clinical trial was carried out with a well-documented probiotic. Thirty-two infants were enrolled, 16 in the probiotic group and 16 in the placebo group. Urine samples were collected from each subject before starting supplementation and at the end of the study period. Metabolomic profiles were obtained using a gas chromatography/mass spectrometry instrument. Subsequently, to compare groups before and after probiotic supplementation, univariate and multivariate statistical analysis were performed.

RESULTS: In the *L. reuteri* treated group all metabolites for all class of nutrients (sugars, amino acids, carboxylic acids) resulted more abundant after the study period. The comparison with a control group (placebo treated), confirmed this effect on urines.

CONCLUSIONS: The metabolomic analysis of urine samples from infants treated with *L. reuteri* DSM 17938 allowed to detect some interesting features related to the effect of this treatment on urinary metabolome. To validate the results, a test on a larger cohort is required.

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KEY WORDS: Lactobacillus reuteri; Colic; Urine; Gas chromatography-mass spectrometry. metabolomics.

Infantile colic was defined by Wessel[1] as full force episodes of infantile crying for more than 3 hours a day at least 3 days a week for 3 weeks or more in an otherwise healthy newborn. Recently, the Rome IV Committee modified the definition as follow: parents have to report that their infant has cried or fussed for 3 or more hours per day, during 3 or more days in the preceding week.[2] Infantile colic is a common condition, since it affects about 30% of otherwise healthy infants.[3] Immature or dysfunctional intestinal microbiota may lead to a low-grade inflammation and abnormal intestinal metabolism, resulting in colic symptoms.[4] The etiology of infantile colic is multifactorial and not fully understood but gut dysmotility and visceral hyper-sensitivity are regarded as main factors behind the condition, and growing evidence

in the literature has also linked the gut microbiota to colicky subjects. Newborns with colic showed lower microbiota diversity and stability in their first weeks of life.[5] Low levels of *Lactobacillus* and *Bifidobacterium* and high levels of coliform bacteria, in particular *Escherichia coli*, have been observed in colicky infants compared to non-colicky ones. Such imbalance could be a possible cause for abnormal gut motility and increased gas production, implicating dysbiosis as a key driver in infantile colic.[6] *Lactobacillus reuteri* DSM 17938 is, until now, the only probiotic with expert recommendations for treatment of colicky breastfed infants. Possible mode of actions of *L. reuteri* includes an improvement of gut motility and direct effect on visceral pain. In addition, it has also been shown that supplementation of *L. reuteri* in colicky babies increased fecal *Lactobacilli* and decreased fecal *E. Coli*. These changes in the microbiota may also be one of the explanations behind the clinical improvement of colicky infants receiving probiotics. However, the mechanism of action of probiotics is not fully clarified as the possible changes in metabolism connected to probiotic supplementation.[7] Metabolomics produces a profile of small molecules derived from cellular metabolism and can directly reflect the outcome of complex networks of biochemical reactions, thus providing insights into multiple aspects of host response and physiology.[8] Thus far, metabolomic studies on the effects of probiotic supplementation regarded mainly fecal samples analysis joined with the fecal microbiota study.[9-11] In the present study we examined urines from subjects treated with a randomized double blind controlled study with *Lactobacillus reuteri* DSM 17938 to investigate the effects of probiotics on urinary metabolites by using a global unbiased mass spectrometry-based platform coupled with gas chromatography.

Materials and Methods

Study population

We carried out a randomized study from August 2017 to September 2019. Thirty-two colicky breastfed infants, aged 15-60 days, were enrolled at the outpatient's Department of Pediatrics of the University of Turin-Regina Margherita Children's Hospital–A.U.O. Città della Salute e della Scienza di Torino. Inclusion criteria were as follows: infants aged between 28 and 61 days, gestational age between 37 and 42 weeks, birth weight between 2500 g and 4000 g, and exclusively breastfed. Subjects were excluded if they had clinical evidence of chronic illness or gastrointestinal disorders or if they received antibiotics or probiotics in the week preceding recruitment. The study population characteristics are reported in Table I.

Outcomes

The primary outcome was to analyze the urinary metabolites in a group of newborns with colic at the time of allocation and after supplementation for 30 days with *L. reuteri* DSM17938 or the placebo.

The secondary outcome was to evaluate the changes in crying time per day, after administration of the probiotic or the placebo. Daily crying and fussing times were recorded in a structured diary, and maternal questionnaires were completed to monitor changes in infant colic symptoms and adverse events.

Colic was diagnosed according the Wessel definition revised by Rome IV criteria,[1, 2] *i.e.* when infants had at least 3 episodes of unexplained full force crying lasting more than 3 hours per day on at least 1 days a week. All parents provided written informed consent to the inclusion of their infants in the study. The protocol was approved

TABLE I.—*Characteristics of the two study groups of infants:* L. reuteri *or placebo treated.*

Variable	*L. reuteri* (N.=16)	Placebo (N.=16)	P value
Gender (M/F)	11/5	9/7	>0.05
Age at recruitment (mean days, range)	43 (28-58)	41 (32-61)	>0.05
Delivery (vaginal/caesarean)	10/6	11/5	>0.05
Birth weight (grams)	3300 (2550-3970)	3270 (2620-3960)	>0.05
Crying time (mean daily minutes per day, standard deviation)	268±6	259±43	>0.05

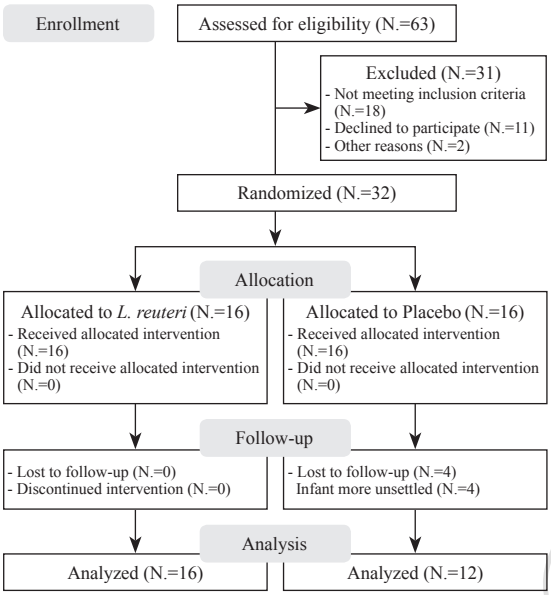

Figure 1.—Study flow diagram.

by the Ethics Committee of the Azienda Ospedaliera, OIRM S. Anna–Ospedale Mauriziano in Turin, Italy (prot 205/CEI, PRAT 75).

Trial was registered at clinical gov, n. NCT 00893711.

Study design and samples collection

The flow diagram of the study is reported in Figure 1.

Thirty-two enrolled infants were randomly assigned to receive *L. reuteri* DSM 17938 or placebo by a computer-generated randomization list. Randomization was done using the random-digit method, based on computer-generated numbers by a statistician blinded after assignment. It was used a 2-treatment randomization scheme with random block of varying size (Stata 9 [Stata Corp, College Station, TX, USA] Ralloc procedure). The assignment was done by someone not involved in any other part of the study. Active probiotic study product (manufactured by Noos s.r.l. company, Rome, Italy) contained a suspension of freeze-dried 108 colony forming units (CFU) of *L. reuteri* DSM 17938 in a mixture of sunflower oil and medium-chain triglyceride oil supplied in a 5 mL dark bottle fitted with a dropper cap. The placebo was equal in appearance and taste but without active microorganisms. Both preparations were administered in the morning in 5 drops, once a day, 30 minutes before breastfed for a period of 28 days. The flagons were coded and blinded by the statistician for parents and medical doctors, and the code was shown to the principal researchers once recruitment, and all laboratory and statistical analyses were widespread. At enrollment, each infant underwent a medical examination by a pediatrician (F.S.) and parents completed a questionnaire to obtain data concerning type of delivery, birth weight and gestational age. The pediatrician asked the parents to account full force crying using a structured day diary before enrollment. The mean daily minutes of crying were described by parents and calculated as a sum of crying and fussing with crying each day. Urines were collected into a sterile Eppendorf tube during the first day of the study period (before receiving any kind of treatment) and at the end of the treatment, and were immediately stored at -80 °C. Samples were sent to the University of Cagliari to be analyzed by the GC-MS (gas chromatography-mass spectrometry) platform at the Department of Chemical and Geological Sciences.

Samples preparation and GC-MS analysis

Urines were analyzed as previously reported.[12] In brief, 150 µL of urine were treated with 800 µL of urease solution (1 mg/mL), 800 µL of cold methanol was added, and the mixture was centrifuged at 4 °C, 14000 rpm for 10 minutes 1200 µL of supernatant were evaporated to dryness, treated with 30 µL of a 0.24 M solution of methoxylamine hydrochloride in pyridine and left to react for 17 hours at room temperature; 30 µL of *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were added and the mixture was left to react for 1 h at room temperature. The samples were diluted with 600 µL of hexane containing tetracosane (0.01 mg/mL) as internal standard, just prior to GC-MS analysis. Samples were analyzed using a MS Agilent 5975C interfaced to the GC 7820 equipped with a DB-5ms column (J & W). Each chromatogram was analyzed using the free software AMDIS (Automated Mass Spectral Deconvolution and Identification System; http://chemdata.nist.gov/mass-spc/amdis). Successively, each peak was identified by comparing rela-

tive mass spectrum and retention time with those stored in an in-house made library including 296 metabolites. Other metabolites were identified using NIST08 (National Institute of Standards and Technology's mass spectral database), and Golm Metabolome Database (GMD; http://gmd.mpimp-golm.mpg.de/). This strategy allowed for the detection of 90 compounds: following the identification levels defined by the Metabolomics Standards Initiative (MSI)[13] 73 were "confidently identified compounds" (level 1), 16 "putatively annotated compounds" (level 2) and 1 "putatively annotated compound class" (level 3). AMDIS analysis produced an electronic sheet data matrix (Microsoft® Excel®, Microsoft Co, Redmond Washington DC, USA) that was submitted to univariate and multivariate statistical analysis.

Statistical analysis

The AMDIS data matrix including 90 metabolites was processed with the integrated web-based platform MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/).[14] Missing values were replaced with the half of the minimum positive values in the original data, and after normalization by sum, data were log transformed, and Pareto scaled. Univariate analysis, partial least square discriminant analysis (PLS-DA) and its associated variable importance in projection (VIP) score were performed. PLS-DA models were tested with the Leave-one-out cross validation (LOOCV) method for the evaluation of statistical parameters (accuracy, correlation coefficient R2, cross validation coefficient Q2),[15] which allowed determining the optimal number of components for the model description. Power analysis allowed to calculate the sample size required to reach a typical predicted power of 0.8. The significance criterion used was a False Discovery Rate (FDR) value of 0.1.

The normal distribution of the crying time per day has been tested using the Kolmogorov-Smirnov test (K-S test). This analysis endorses that crying times per day are normally distributed in both groups of infants (*L. reuteri* and placebo) at Day 0 and at Day 28 (K-S Test p>0.05). For crying time data are reported as mean±standard deviation.

Results

Applying a paired T-test, we observed that mean daily crying time of the day before enrollment was significantly higher than after the study period after *L. reuteri* DSM 17938 supplementation (268±46 minutes vs 115±31 P<0.01). The differences between the means of crying time in the placebo group at Day 0 (259±43) and the means variable at Day 28 (236.8±39) was not statistically significant (P=0.121).

L. reuteri treated group

Urines from 16 infants treated with *L. reuteri* DSM 17938 were analyzed: each infant provided two samples, one before treatment and one at the end of the study period. Univariate analysis (T-test) revealed 23 metabolites statistically significant in the comparison between urines from infants before and after *L. reuteri* DSM 17938 supplementation (Table II).

All metabolites reported in Table II resulted more abundant in the infants after treatment with

TABLE II.—*Statistically significant (False Discovery Rate FDR<0.05) metabolites from univariate analysis (T-test) of the infants before and after* L. reuteri *DSM 17938 treatment. All reported metabolites resulted more abundant in the infants after treatment with* L. reuteri *DSM 17938.*

Name	P-value	FDR
Ribose	0.0013028	0.033344
Glycolic acid	0.0016392	0.033344
Threitol	0.0017052	0.033344
Histidine	0.0026629	0.033344
Galactitol	0.0037128	0.033344
Threonic acid	0.0042697	0.033344
cis-Aconitic acid	0.0044084	0.033344
Arabitol	0.0045041	0.033344
Citric acid	0.0047295	0.033344
3-Hydroxybutyric acid	0.0047634	0.033344
Glycyl-proline	0.0053066	0.033769
2-Hydroxyisobutyric acid	0.0066364	0.038397
3,4,5-Trihydroxypentanoic acid	0.0071308	0.038397
4-Hydroxyphenylacetic acid	0.0077142	0.038571
Galactonic acid	0.0099591	0.041992
Lyxonic acid	0.010342	0.041992
Xylose	0.010785	0.041992
Erythronic acid	0.011083	0.041992
Pseudouridine	0.011398	0.041992
4-Deoxyerythronic acid	0.013037	0.0441
Creatinine	0.01323	0.0441
Erythritol	0.014875	0.045844
Glutamine	0.015063	0.045844

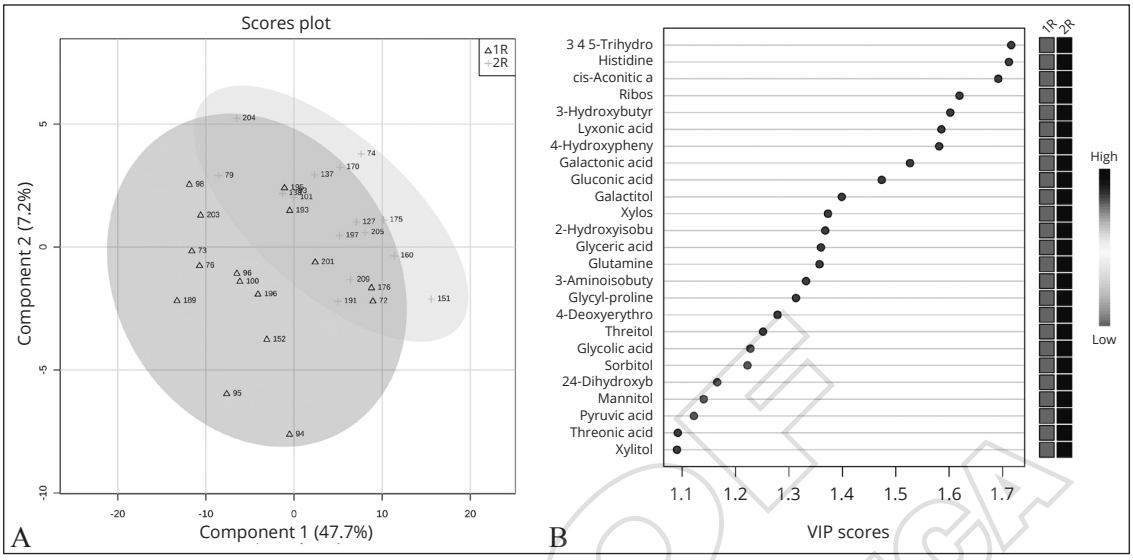

Figure 2.—A) 2D scores plot showing PLS-DA discrimination between urine samples of infants with colics before treatment (group 1R, red in the online version) and urine samples of the same infants after L. reuteri DSM 17938 treatment (group 2R, green in the online version). The shaded areas indicate the 95% confidence regions; B) summary plot showing the most important metabolites ranked based on the variable importance in projection (VIP) score. The mini heatmap on the right indicate their concentration variations within the groups (group 1R, before treatment; group 2R, after treatment).

L. reuteri DSM 17938. They belong to different chemical classes, namely sugars and reduced sugars (ribose, threitol, galactitol, arabitol, xylose, erythritol), amino acids (histidine, glutamine, plus the dipeptide glycyl-proline), carboxylic acids (glycolic, threonic, *cis*-aconitic, citric, 3-hydroxybutyric, 2-hydroxyisobutyric, 3,4,5-trihydroxypentanoic, 4-hydroxyphenylacetic, galactonic, lyxonic, erythronic, 4-deoxyerythronic) and amines (pseudouridine, creatinine). The supervised PLS-DA analysis of the two groups of pre-treatment and post-treatment samples allowed to obtain the model reported in Figure 2.

This model was best described by the first principal component, showing accuracy=0.65625, R2 (goodness of fit)=0.29903, and Q2 (goodness of prediction)=0.1547. The cross validation (CV) of the model returned a performance measure of p=0.09, probably due to the low number of samples. The metabolites resulting most responsible for the discrimination between the two groups are reported, in order of importance, in Figure 2B.

As evident from Figure 2B, all metabolites were found in greater amount in the urines of infants after probiotic treatment. They are roughly analogous to those revealed by the univariate analysis, as can be expected. The power analysis reported a sample size of 100 per group, *i.e.* 100 infants should be treated with the probiotic.

Placebo treated group

The group of infants treated with the placebo was initially formed by 16 infants, equal to the *L. reuteri* DSM 17938 treated group. Unfortunately, only 12 attended to the follow-up visit at the end of the treatment period, reducing the size of the control group. The univariate analysis did not reveal any metabolite significantly different between the group pre-placebo treatment and post-placebo treatment. The PLS-DA analysis allowed to obtain the model reported in Figure 3.

This model was best described by the fifth principal component, showing accuracy=0.55, R2=0.98259, and Q2=-0.048562. Unfortunately, the model did not reach statistical significance (P=1), due to the low number of samples. Nonetheless, the trend showed by the most important metabolites, reported in Figure 3B, revealed some compounds more abundant in urines at the end of the study period: carboxylic acids (succinic, threonic, lactic, glyceric), amino acids (threonine, 4-hydroxyproline, proline, glutamine), reduced sugars (erythritol, ribitol). Other

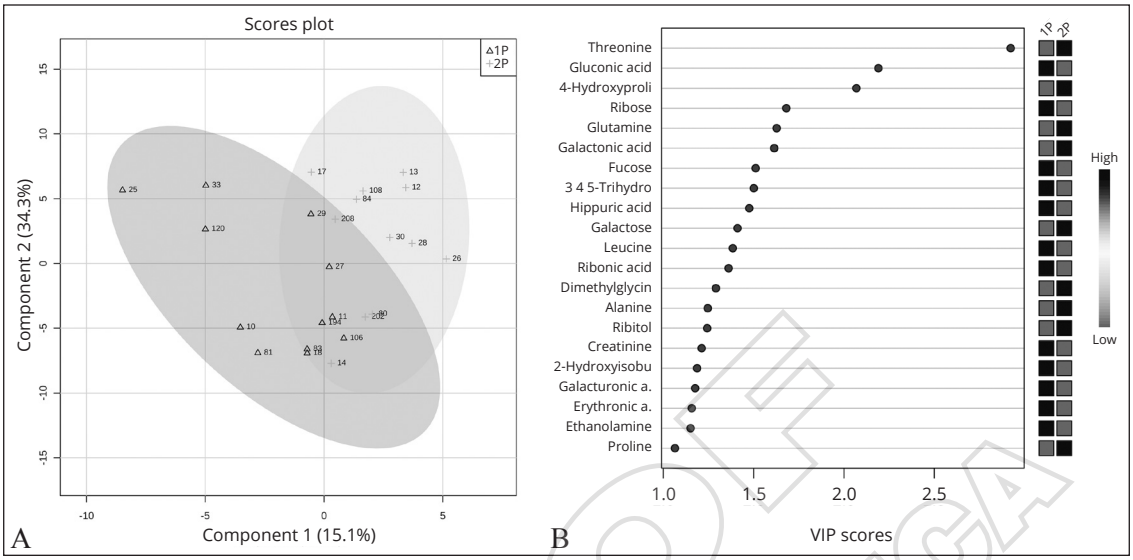

Figure 3.—A) 2D scores plot showing PLS-DA discrimination between urine samples of infants with colics before placebo treatment (group 1P, red in the online version) and urine samples of the same infants after placebo treatment (group 2P, green in the online version); B) summary plot showing the most important metabolites ranked based on the variable importance in projection (VIP) score.

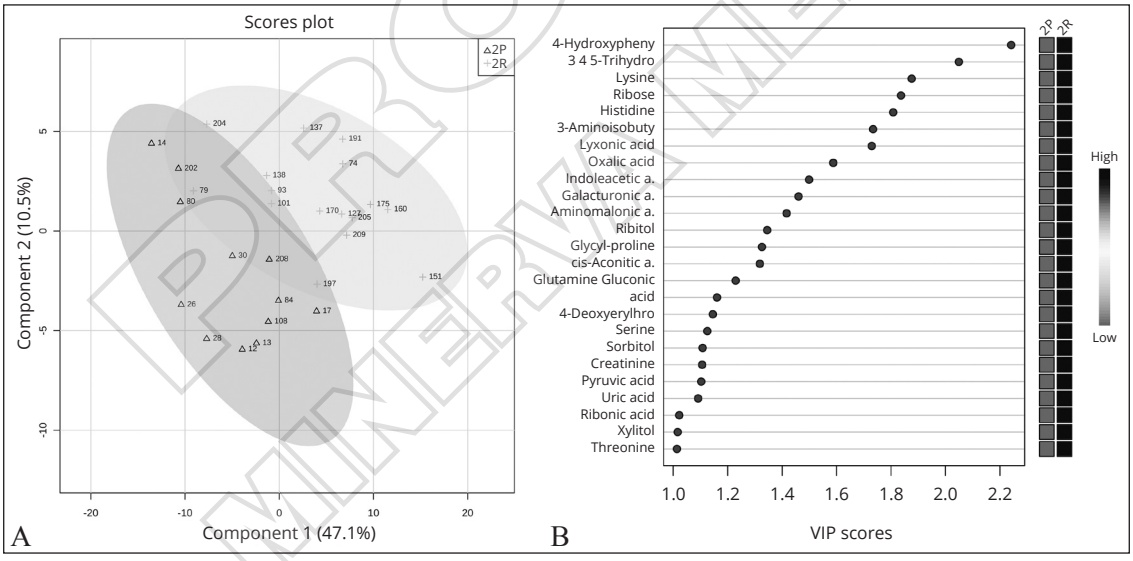

Figure 4.—A) 2D scores plot showing PLS-DA discrimination between urine samples of post-placebo treatment infants (group 2P, red in the online version) and samples of post-L. reuteri treatment infants (group 2R, green in the online version); B) summary plot showing the most important metabolites ranked based on the variable importance in projection (VIP) score.

metabolites were found in lower amount at the end of this period: N-(4-hydroxybenzoyl)glycine (hippuric acid derivative), gluconic acid, arabinose, fucose, leucine, 3,4,5-trihydroxypentanoic acid, creatinine, malic acid. The power analysis gave a sample size of 200 per group, but the low perturbation detected between samples allowed to reach a very low predicted power of 0.4.

Post-placebo group *vs.* post-*L. reuteri* group

To further investigate the metabolic difference between infants treated with the probiotic and

those treated with the placebo, we compared these groups at the end of the treatment period, obtaining the PLS-DA model summarized in Figure 4.

This model was best described by the third principal component, showing accuracy=0.76923, R2=0.87226, and Q2=0.30537. The CV test scored P=0.42, due to the low number of samples, as in the above reported comparisons. Despite the low statistical significance of the model, the metabolites trend showed in Figure 4B allowed to draw some interesting insights. All metabolites resulted more abundant in urines from probiotic treated infants, regardless of their chemical class. It can be assumed that each class of nutrients took advantage of improved gut absorption, showing a clear effect of the probiotic treatment in comparison with the placebo. The power analysis, as expected, gave the same results as for the comparison between pre-and post-*L. reuteri* treatment.

Pretreatment *vs.* post-placebo *vs.* post-*L. reuteri*

A three groups analysis examined urine samples from all infants at the beginning of the study and before any treatment (32), the post-placebo treatment group (12), and the post-*L. reuteri* treatment group (16). The univariate analysis ANOVA (Analysis of Variance) revealed eight statistically significant metabolites (Table III).

TABLE III.—*Statistically significant (False Discovery Rate FDR<0.05) metabolites from univariate analysis (ANOVA) of urines from infants before any treatment, placebo treated, and* L. reuteri *DSM 17938 treated. All reported metabolites resulted more abundant in the infants after treatment with* L. reuteri *DSM 17938.*

Name	P-value	FDR
4-Hydroxyphenylacetic acid	6.6733E-5	0.0044711
3,4,5-Trihydroxypentanoic acid	1.909E-4	0.0063952
3-Aminoisobutyric acid	0.0021784	0.035773
Ribitol	0.0022971	0.035773
cis-Aconitic acid	0.0033656	0.035773
Glutamine	0.0036234	0.035773
Lyxonic acid	0.0040867	0.035773
Uric acid	0.0042714	0.035773
4-Deoxyerythronic acid	0.0056547	0.042096
Ribose	0.006629	0.044251
Threonine	0.0075546	0.044251
Histidine	0.0079255	0.044251

All the reported metabolites were found in higher amount in urines of probiotic treated infants at the end of the treatment period.

The PLS-DA model summarized in Figure 5 was best described by the second principal component, showing accuracy=0.59615, R2=0.50473, and Q2=0.17002. This model reaches statistical significance with P<0.01.

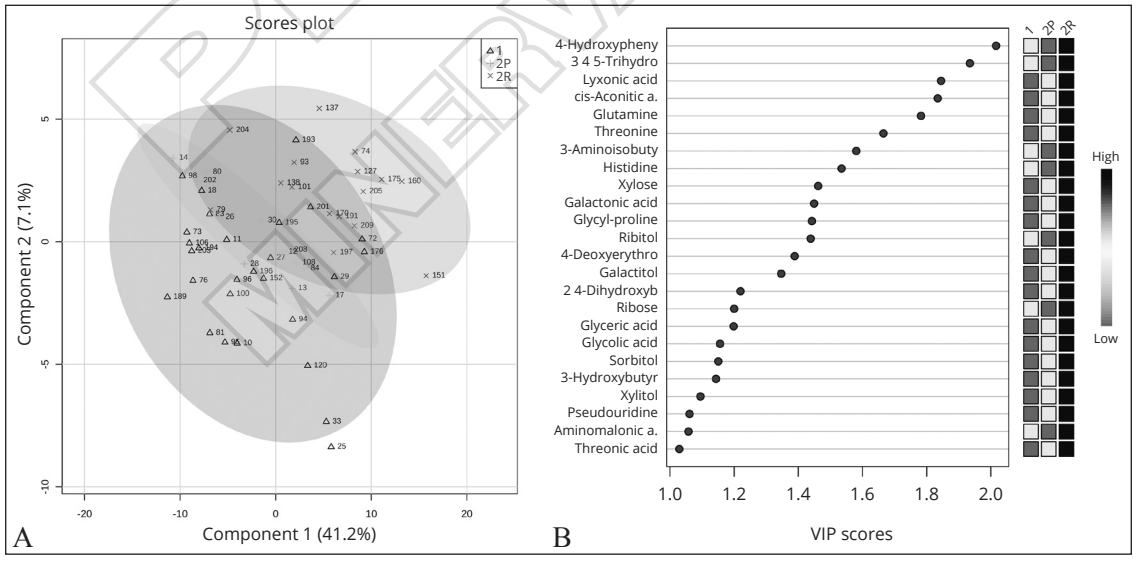

Figure 5.—A) 2D scores plot showing PLS-DA discrimination between urine samples of infants before any treatment (group 1, red in the online version), post-placebo treatment (group 2P, green in the online version) and post-*L. reuteri* treatment (group 2R, violet in the online version); B) summary plot showing the most important metabolites ranked based on the variable importance in projection (VIP) score.

Figure 5B clearly shows all metabolites more abundant in urines after the probiotic treatment, in agreement with the previous comparisons. The metabolites trend in pre-treatment (1) and post-placebo treatment (2P) samples shows an uneven behavior, being some metabolites more abundant in pre-treatment samples than in post-placebo samples (4-hydroxyphenylacetic acid, 3,4,5-trihydroxypentanoic acid, lyxonic acid, histidine, 3-aminoisobutyric acid, ribitol, ribose, uric acid). All other metabolites followed the increasing trend pre-treatment/post-placebo treatment/ post-probiotic treatment.

Discussion

Despite the exponential growth of literature examining and describing the gut microbiome, it is still the most poorly understood aspect of the biological drivers affecting gut function and health in early infancy.[16] Urine-based metabolomic researches play a promising role in the fields of pediatrics and neonatology, relying on simple and non-invasive collection procedures while integrating a variety of factors such as nutrition, genotype and diseases.[12, 17] Metabolomics is defined the quantitative analysis of a large number of low molecular weight metabolites that are intermediate or final products of the metabolic pathways in a living creature. Each metabolic profile detectable in a human biological fluid are produced by the interaction between gene expression and the background.[18]

Many factors including abnormalities in gastrointestinal motility, visceral sensation, brain-gut interaction, psychosocial distress, gut immune activation, intestinal permeability and intestinal and colonic microbiome have been suggested to play a role in the pathogenesis of infantile colic.[4, 5]

Until now there is no diagnostic biomarker clearly related to infantile colic: the diagnosis relies only on the identification of related symptoms and the exclusion of other organic conditions common in early infancy.[19]

Metabolomics has increasingly been applied for identification of mechanistic, diagnostic and/or prognostic metabolites in adult patients with digestive diseases including inflammatory bowel disease such as with Crohn's disease and ulcerative colits.[20]

The metabolomic analysis of urines from infants treated with *Lactobacillus reuteri* DSM 17938, allowed to detect some interesting features related to the effect of this treatment. The comparison of urines from the same infants pre- and post-*L. reuteri* treatment showed an increase of all metabolites as evidenced by univariate and multivariate analysis. This result may be related to the improved gut absorption of all class of nutrients (sugars, amino acids, carboxylic acids). To investigate if this effect was truly due to the probiotic supplementation, rather than to the physiologic improvement of gut absorption over time, we examined urines from infants treated with a placebo. The comparison between urines of infants pre- and post-placebo treatment did not reveal a clear increasing trend, being some metabolites more abundant in the pre-placebo and others in the post-placebo samples. To add other pieces to the jigsaw, the comparison between two groups of infants, post-placebo and post-probiotic treatment, far from reaching a full statistical significance, allowed to confirm the difference in the nutrients absorption (and urinary excretion) clearly promoted by the *L. reuteri* supplementation. The comparison between three groups of urine samples, from infants before any treatment, post-placebo and post-probiotic treatment, allowed to confirm that the *L. reuteri* supplementation promotes a more efficient and quick recovery of the gut absorption, possibly related to infant colics. Of interest, Greene *et al.* recently reported that specific gut microbiota and associated gastrointestinal morphologies support nutrient extraction from challenging resources, perhaps ultimately facilitating host species' diversity and specialized feeding metabolism.[21] In order to optimize the use of probiotics particularly in the first period of life, a more complete biochemical understanding of the impact that these supplements have on the community and functioning of the gut microbiota is required. Nutrients such as monosaccharides and the microbiome may have a deep impact on the brain by influencing its development and function in health and disease. However, the mechanisms by which they shape brain function have only started to be uncov-

ered.[22] Considering that growing evidences have reported that infants with colic, treated with *L. reuteri* DSM 17938, have a clinical improvement and also a significant reduction on fecal calprotectin levels,[23] our data support the possibility of the observed better utilization of nutrients could be linked to the reduction of the gut inflammation and to a more favorable gut- host metabolism.[4] Further, the effects of low fermentable oligosaccharides, disaccharides, monosaccharides and polyols such as (FODMAP) are known with regard to their impact on microbiota and on the evolution of the gastrointestinal disease, particularly in adults.[24] Currently, evidence of the effectiveness of dietary modifications for the treatment of infantile colic is scanty and at significant risk of bias, since the few available studies had small sample sizes, and most had serious limitations.[25] Our knowledge about infantile colic is still only partial and it is important to understand the different aspects of potential contributions of the microbiota to pathophysiology of colicky in order to identify a personalized remedy.

Limitations of the study

The main limitation of this study lies in the lack of a healthy breastfed infants group and in the consequent possibility to correlate data from gut microbiota analysis to urinary metabolomic results. Moreover, the low number of samples collected do not allow the validation of more definitive conclusions. The calculated minimum sample size to obtain statistically powerful results is of 100, corresponding to 100 colicky infants to be treated with the probiotic, and possibly to be compared with the same number of matched healthy controls.

Carefulness should be taken in considering the metabolomic data obtained from the present randomized study, and larger double-blind placebo studies are necessary to provide additional information.

Conclusions

To the best of our knowledge, this is the first study aimed to evaluate urinary metabolites of infants with colic before and after probiotic supplementation with *L. reuteri* DSM 17938 using a gas chromatography-mass spectrometry platform. Despite the small sample size, our study reported that *L. reuteri* DSM 17938 treatment is linked to an increase of urinary metabolites and may be related to gut absorption improvement. Our findings allowed to detect some interesting features related to the effect of this treatment. All metabolites belonging to all class of nutrients (sugars, amino acids, carboxylic acids) resulted more abundant in infants after the probiotic treatment. The same trend was observed in comparison with the placebo treated subjects, suggesting a more effective improvement of gut absorption in the probiotic treated group. To validate these results, new studies on a larger cohort of infants are required.

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