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DIFFERENCES IN COMPOSITION OF GUT MICROBIOTA IN WOMEN WITH AND WITHOUT HYPEREMESIS GRAVIDARUM

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Abstract – *Objective:* Previous research has reported associations between *Helicobacter pylori* and hyperemesis gravidarum (hyperemesis). Whether the human gastrointestinal microbiota is associated with hyperemesis has not been studied. The aim of the present study was to explore gut microbiota among women with and without hyperemesis. In addition, we also studied the consequence of the absence or presence of *H. pylori* on the metagenomics of intestinal microbiota.

Patients and Methods: Faecal samples were collected from immigrant pregnant women in Norway hospitalised due to hyperemesis (n=52) and pregnant women without hyperemesis (n=161). DNA extraction, 16S rRNA gene amplification and sequencing, and *H. pylori* PCR were conducted. Data on maternal age, parity, number of pregnancies, or patient history regarding hyperemesis was registered. Furthermore, information on country of birth and length of stay in Norway was also recorded. Additionally, use of antibiotics during the last 2 months before inclusion in the study was registered.

Results: Results from the *H. pylori* specific PCR demonstrated a lower carriage rate among pregnant women without hyperemesis (23.5%) compared to the hyperemesis-positive group (26.9%). However, the difference between the two groups was not significant ($p>0.05$). Faeces from hyperemesis-negative women were comprised of the following groups of bacteria (N=9): *Actinobacteria* (2 groups), *Bifidobacteriales, Bifidobacteriaeae, Bifidobacterium, Bulleidia, Clostridiacae, Clostridiae div, and Unclassified Clostridiaceae*. A higher number of groups (N=20) was detected in faeces from hyperemesis patients (viz., *Alcaligenacae, Bacterioidaceae, Bacterioides, Betaproteobacteria, Burkholderidales, Clostridia, Clostridiales* other types, *Firmicutes, Ocillospira* other types, *Parabacteroides, Porphyromonadaceae, Rikenellaceae, Rikenellaceae* other types, *Ruminococcacae, Ruminococcacae, Ruminococcaceae* other types, *Ruminococcus, Sutterella,* unclassified *Lachnospiraceae, and* unclassified *Rikenellaceae*).

Conclusions: In hyperemesis-positive women, gut microbiota displayed higher alpha diversity than hyperemesis-negative women ($p=0.0015$). *Clostridiales* were present in women with and without hyperemesis. However, more groups were observed in the hyperemesis group (viz., *Bacteriodaceae, Bacteroides, Firmicutes, Clostridia* and *Betaproteobacteria*).

Keywords: Pregnancy, Hyperemesis gravidarum, Faeces microbiota, *Helicobacter pylori*, Case-control study.

INTRODUCTION

Hyperemesis gravidarum (hyperemesis) is characterised by excessive nausea and vomiting during early pregnancy and affects between 0.3% and 10.8% of women[1]. Due to nutritional deficiencies and dehydration, hyperemesis is a common cause of admittance to hospital during first and second trimesters and is associated with adverse pregnancy and birth outcomes, such as placental dysfunction disorders and small for gestational age (SGA) children. Its aetiology remains a puzzle. Hyperemesis clusters in families and is inherited through the maternal line from mothers to daughters, suggesting genetic or epigenetic contributing factors[2]. Recent studies have found genes encoding placental proteins, such as GDF 15 and IGFBP7, and hormone receptors, such as GFRAL and PGR, to be involved[1]. A recent systematic review and meta-analysis on diagnostic markers for hyperemesis gravidarum reported that *Helicobacter pylori* (*H. pylori*) detection by looking for *H. pylori* specific IgG antibodies might be useful in specific patients. By contrast, several studies found no such correlation, with most women being seropositive for *H. pylori* not having hyperemesis[1]. Other studies have revealed alterations in microbiota composition in patients suffering from various diseases according to *H. pylori* status[3]. In general, gut microbiota is known to modulate the pathogenesis, as well as treatment of diseases[4]. In uncomplicated pregnancies, the diversity of gut bacteria is known to decline between first and third trimesters[5,6], when the microbiota changes to resemble those people who are obese or who have metabolic syndrome. This means that the proportions of *Proteobacteria*, which are associated with inflammation, and *Actinobacteria*, which is known to degrade specific fibres, increase during pregnancy[6,7]. Additionally, gut microbiota is also known to change during severe disease and failure to eat[6-9]. Whether human gastrointestinal microbiota is associated with hyperemesis has not yet been studied. The aim of the present study was to explore gut microbiota among women with and without hyperemesis using faecal samples collected from pregnant women diagnosed with and without hyperemesis. In addition, we wanted to study the consequence of absence or presence of *H. pylori* on the metagenomics of intestinal microbiota.

MATERIAL AND METHODS

Data from participants, who were all immigrant women in Norway, including maternal age, parity, number of pregnancies, or whether they had experienced hyperemesis previously was recorded. Furthermore, data regarding country of birth plus length of stay in Norway were obtained. Additionally, use of antibiotics during the last 2 months before inclusion in the study was registered.

Faeces samples were collected from 52 hyperemesis inpatients and 161 healthy controls without hyperemesis in the outpatient ward at the time point for ultrasound screening between the 17th and 22nd gestational weeks. The faeces obtained were stored at -20°C after an overnight stay in a refrigerator at +4°C for hyperemesis patients. Faeces samples from controls were sent by mail in transport containers without any additives and kept frozen (-20°C) after arrival in the laboratory until analyses were performed.

DNA Extraction, 16S rRNA Gene Amplification and Sequencing

DNA extraction was done using the QIAamp DNA stool Mini Kit (Cat. No. 51504) according to the manufacturer's instructions (Qiagen, Switzerland). The 16S rRNA gene amplification was conducted by targeting variable regions 3 and 4 with an expected product size of ca 450 base pairs using primers 341f and 805r (Table 1). The PCR was performed as described[10,11] using Pfu Ultra High-fidelity DNA polymerase (Cat. No. 600384) and PurePeak dNTPs (Cat. No. NU606001). The PCR products were checked on a 1% agarose gel for specific product confirmation.

For the nested PCR, the same primers were used together with an adaptor and barcode sequence (Table 2). The products were purified using Agencourt AMPure XP (Cat. No. A63881) and quantified with Pico green (Cat. No. P7589) on a LC480 (Roche, Basel, Switzerland).

TABLE 1. THE 16S RRNA GENE AMPLIFICATION BY TARGETING THE VARIABLE REGIONS 3 AND 4.

Primer	Sequence 5'→3'	Source
341f	CCTACGGGNGGCWGCAG	2
805r	GACTACHVGGGTATCTAATCC	2
Adaptor B 341f	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCCTACGGGNGGCWGCAG	2, 5, 6
Adaptor A 805r MID1	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCGTGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID2	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCTCGACAGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID3	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACGCACTCGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID4	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID5	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID6	CCATCTCATCCCTGCGTGTCTCCGACTCAGATATCGCGAGGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID7	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTGTCTCTAGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID8	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCGTGTCGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGTATCAGCGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID10	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCTATGCGGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATACGTCTGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID12	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTGAGCTAGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID13	CCATCTCATCCCTGCGTGTCTCCGACTCAGCATAGTAGTGGACT ACHVGGGTATCTAATCC	2, 5, 6
Adaptor A 805r MID14	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGAGATACGACT ACHVGGGTATCTAATCC	2, 5, 6

Note: The 16S rRNA gene amplification was done by targeting the variable regions 3 and 4 with an expected product size of ca 450 base pair using primers 341f and 805r.

Next generation sequencing (NGS; Roche GS Junior) was performed by pooling 14 samples using different multiplex identifiers (MIDs) in order to split the sequencing data afterwards[12,13].

16S rRNA Gene Sequencing Data Analysis

The analysis of sequencing data was conducted using Quantitative Insights into Microbial Ecology (QIIME) version 1.9.1[14]. Reads of quality lower than 25, lacking a barcode, and/or shorter than 200 or longer than 1000 nt were not analysed further. The remaining reads were assigned to samples based on their nucleotide barcodes. Denoising of sequences and clustering to operational taxonomic units (OTU) using a 97% sequence identity threshold was performed using USEARCH v6.1.544[15]. The representative OTUs were aligned against the Greengenes core set database[16] using PyNAST[17] with a default minimum identity of 75%. Taxonomy was assigned to aligned sequences using UCLUST[15]. A phylogenetic tree was generated in FastTree[18] after filtering the alignment using a lane mask. The phylogenetic tree was used to build an unweighted UniFrac distance metric[19], which included the calculated distances between samples based on the OTU composition of each sample and visualised using principle coordinate analysis (PCoA). Alpha diversity was calculated at OTU level using the Shannon index based on the average of ten iterations at an equal subsampling size of 363.

TABLE 2. STUDY POPULATION.

Continent of birth	Pregnant women	Hyperemesis	Total
Europe	8	2	10
Africa	48	19	67
Asia	90	21	111
ND	15	10	25
TOTAL	161	52	213
ND=No data available.			
No participants	**Pregnant women**	**Hyperemesis**	**TOTAL**
Mean age (+SD) (yrs)	29.5 (±5.2)	29.3 (±5.2)	
Median No. of births given	1	1	
Median No. of pregnancies	3	2	
% of patients having taken antibiotics during pregnancy	6,4%	20%	
No of years (+SD) in Norway	8.6 (±6.5)	9.5 (±5.9)	
ND	8	5	13

Statistical Analysis

The alpha diversity data were not normally distributed; therefore, the nonparametric, Kruskal Wallis test was used to compare Shannon indices between the groups. UniFrac distances were compared between groups using the permutational multivariate analysis of variance (PERMANOVA) method with 999 permutations, and the results were reported as significant when the *p*-value was lower than 0.05. The comparison of the relative abundance of taxa between groups was performed using the linear discrimination analysis (LDA) effect size (LEfSe) algorithm[20], online Galaxy version. Default statistical parameters of α=0.05 and LDA score 2.0 were used. The comparison of relative abundance of taxa between groups was performed using Linear discriminant analysis Effect Size (LEfSe), online Galaxy version[20].

Real Time-PCR for *H. pylori*

In addition to sequencing, extracted DNA was tested by running Real Time-PCR using a *H. pylori* real time PCR kit (Cat. No. Path-H.pyl-standard, PrimerDesign Ltd, UK), on a light cycler 480 (LC480, Roche) according to the manufacturer's instructions[21]. Two different concentrations of the extracted DNA (2.5 and 25 ng) were applied to run real time PCR to ensure the obtained results were not false negatives due to inhibitors.

RESULTS

We analysed 52 pregnant women affected by hyperemesis and 161 pregnant women without hyperemesis. General analyses were run for all samples, including hyperemesis-positive and hyperemesis-negative ones for group statistics and for the evaluation of factors, such as 'antibiotic use', 'country of birth' and 'years in Norway' (Table 2).

The results from the *H. pylori* specific RT-PCR showed a lower carriage rate among normal pregnant women (23.5%) compared to the group of women suffering from hyperemesis gravidarum (26.9%). However, the difference between the two groups did not reach the level of statistical significance ($p>0.05$) (Table 3).

TABLE 3. RESULTS FROM TESTING FECES FROM PREGNANT WOMEN WITHOUT HYPEREMESIS (NEG) AND WOMEN SUFFERING FROM HYPEREMESIS GRAVIDARUM (POS) BY PRESENCE OF *H. PYLORI* BY RT-PCR.

Hyperemesis	PCR *H. pylori*		TOTAL
	Pos	Neg	
Pos	14 (26,9%)	38	**52**
Neg	38 (23,5%)	123	**161**
			213

Using QIIME, we trimmed, quality filtered, denoised, and picked OTUs and assessed the alpha diversity and the relative abundance in the various groups of individuals at genus level (beta diversity).

H. pylori affected the microbiota composition significantly considering which bacteria were present (unweighted UniFrac), but when taking abundance into consideration (weighted UniFrac), no significant difference in composition was observed. Thus, *H. pylori* was associated with a difference in the types of bacteria present, while the distribution of abundance was similar in the gut flora of ordinary pregnant women (hyperemesis negatives, N=161) (Figure 1).

Further analysis of gut microbiota demonstrated that diversity was higher in women with hyperemesis (p=0.0015) compared to those without hyperemesis. Furthermore, the diversity in hyperemesis patients (blue) was more clustered and displayed a higher average number of OTUs. The hyperemesis-negative women (red) demonstrated dispersal with a larger range of OTUs (Figure 2). As the recovered reads were not abundant, species identification from OTS was not feasible. Thus, we sorted our findings on genus levels linked to the various patient groups.

Faeces from women without hyperemesis included the following groups (N=9): *Actinobacteria* (2 groups), *Bifidobacteriales, Bifidobacteriaeae, Bifidobacterium, Bulleidia, Clostridiacae, Clostridiae div, and* unclassified *Clostridiaceae* (Figure 3).

A higher number of groups (N=20) was detected in faeces from hyperemesis patients (i.e., *Alcaligenacae, Bacterioidaceae, Bacterioides, Betaproteobacteria, Burkholderidales, Clostridia, Clostridiales other, Firmicutes*, *Ocillospira other, Parabacteroides, Porphyromonadaceae, Rikenellaceae, Rikenellaceae Other, Ruminococcacae, Ruminococcacae, Ruminococcaceae other, Ruminococcus, Sutterella*, unclassified *Lachnospiraceae,* and unclassified *Rikenellaceae*.).

Clostridiales were present in both groups, but more bacterial groups were observed in the hyperemesis group, namely, *Bacteriodaceae, Bacteroides, Firmicutes, Clostridia and Betaproteobacteria.* All details are provided in Figure 3.

The women with hyperemesis gravidarum had significantly different microbiota composition compared with the hyperemesis gravidarum negative women (p=0.0015), whereas the presence of *H. pylori* demonstrated no impact on the microbiota composition (p=0.05).

DISCUSSION

Being the first study exploring differences in gut microbiota in women with and without hyperemesis, we found that the microbiota in pregnant women with hyperemesis gravidarum differed from that in pregnant women without hyperemesis. Women with hyperemesis displayed more clustered alpha diversity with higher average numbers of different OTUs compared to pregnant women without hyperemesis.

Initially, we examined the implication of the presence or absence of *H. pylori* in pregnant women without hyperemesis (N=161). Studies on the role of *H. pylori* in patients with hyperemesis and pregnant women without hyperemesis have provided conflicting results. Furthermore, studies from our group have shown conflicting results; that is, one study suggested a correlation in pregnant women born in Africa[22], but another study[23] could not verify findings of associations between the presence of *H. pylori* and hyperemesis. A meta-analysis, however,

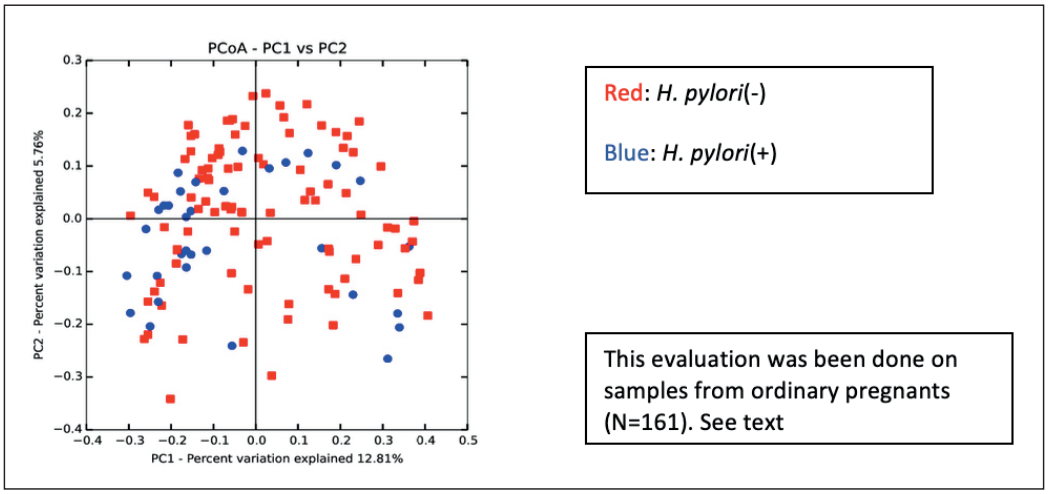

Figure 1. Distribution of taxa in pregnant women with or without carriage of *H. pylori*.

based on 81 studies suggested a relation between *H. pylori* and hyperemesis[24]. It is possible that subsets of cagA might have implications for the occurrence of this condition. However, further examinations, such as those investigating the presence of antibodies against *H. pylori* cagA in patients with the condition, were not attempted in this study. In our study, we observed no difference in faecal microbiota in normal pregnant women whether or not they carried *H. pylori*. This latter finding might be intriguing as stomach microbiota differs according to the presence or absence of *H. pylori* as reported previously[3]. Our findings are, however, in accordance with results from recent studies of flora in various parts of the alimentary tract that have reported no effect of *H.pylori* on the composition of faecal microflora in contrast to significant differences observed in the microbiota of the upper part of the alimentary tract[3].

Recent reports[7-9] highlight that pregnancy alters resident gut microbes in a manner that is thought to facilitate nutrition during pregnancy. In addition, vaginal flora display lower bacterial diversity and an increase in *Lactobacillus* species, *Clostridiales, Actinomycetales,* and *Bacterioidales*. Similar alterations have also been reported in the gut flora of pregnant women[25,26]. These alterations have similarities to the gut flora present in diabetic patients[6]. Species from the genus *Bacterioides* comprise 30% of all bacteria in the gut[4,10,27]. *Bacterioides* spp. may provide complex carbohydrate digesters[27], and a rural diet may lead to enrichment of *Bacterioides* genera (including *Prevotella* and *Xylanibacter*), allowing rural populations to facilitate energy uptake from diets rich in fibres[4]. Similarly, *Actinobacteria* may provide degrad-

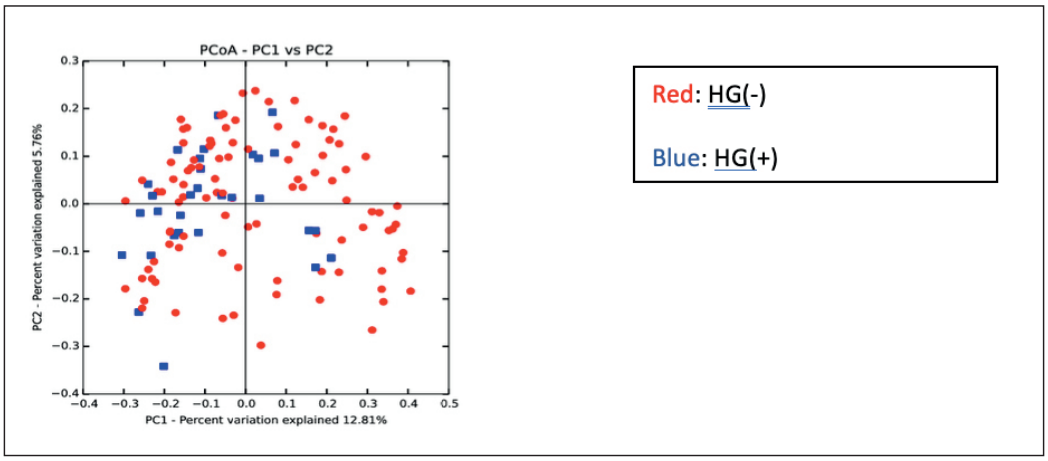

Figure 2. Distribution of taxa in hyperemesis patients vrs pregnant women without hyperemesis.

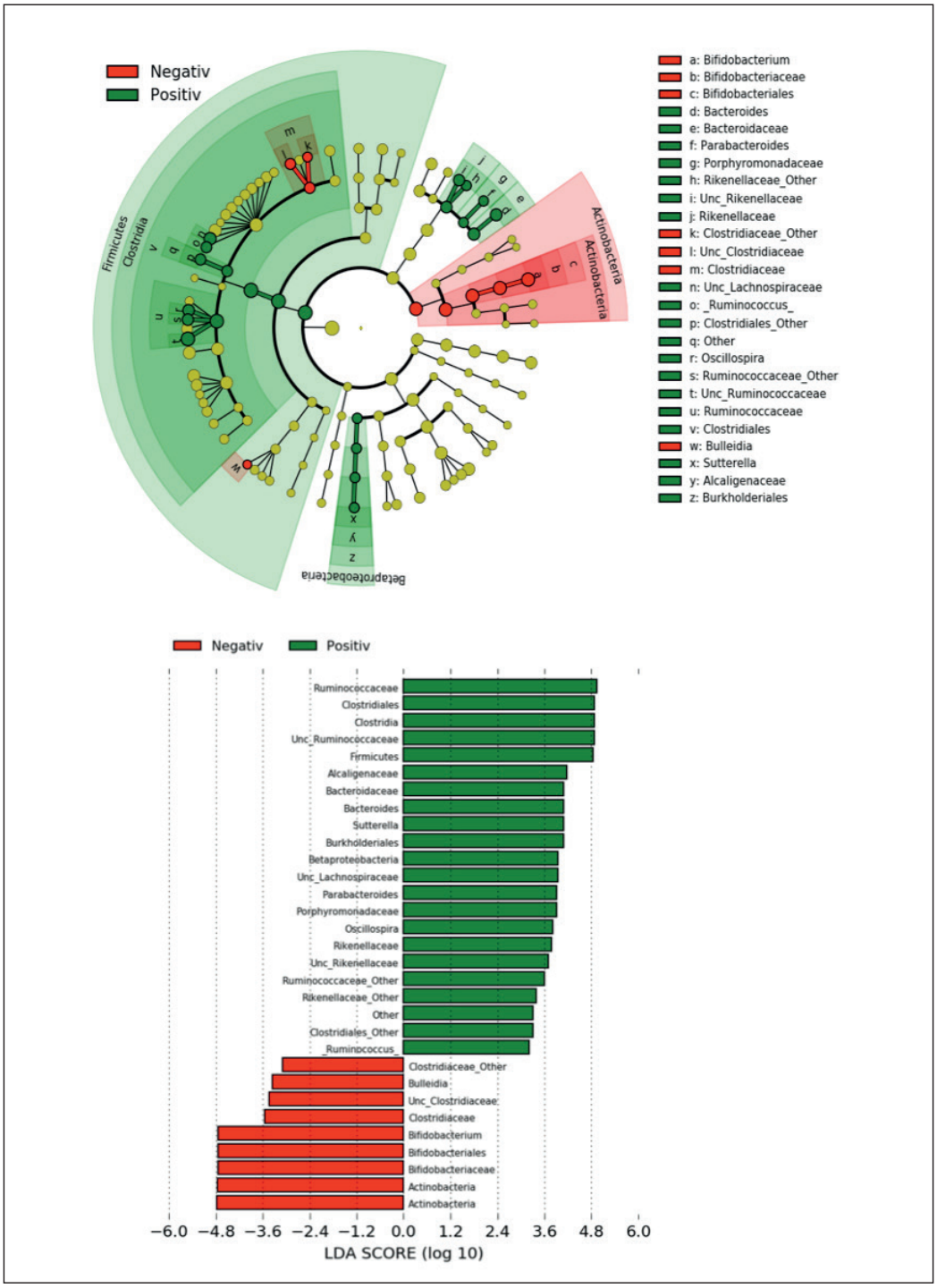

Figure 3. Differential relative abundance analysis hyperemesis (+) Vs (-): (Hyp_Lefse_Hyper. Txt).

ing enzymes for partly digesting fibres. On the other hand, nutrition comprising high saturated fat is associated with reduced richness and diversity in human microbiota. Alpha diversity may be regarded as a predictor of the extent of microbiota change upon the short-term consumption of different protein sources[4]. Usually, in pregnant women, no clinical signs of this change in microbial composition occur. Thus, alterations in gut flora should be expected[26]. We have, however, not found any reports on alterations in women suffering from hyperemesis. Our finding of greater clustered alpha diversity with higher average numbers of different

OTUs in hyperemesis may be a consequence or a contributing factor to the development of hyperemesis. This may be due to new metabolic compounds delivered from presiding gut flora[28]. In our study, *Actinobacteria* were present in women with and without hyperemesis, but several other groups were observed in the hyperemesis group, namely, *Bacteriodaceae, Bacteroides, Firmicutes, Clostridia,* and *Betaproteobacteria*. At present, information concerning microbiota in various patient groups and locations have been published[1,8,29], including studies on the vaginal microbiota of healthy pregnant women[1,5,6,29]. So far, detailed analyses have been presented for various conditions, but we are not aware of any report on the faecal microbiota in women suffering from hyperemesis. The functional role of the different taxa found in women with hyperemesis remains to be elucidated. The shift and increase in number of taxa, namely, *Bacteriodaceae, Bacteroides, Firmicutes, Clostridia,* and *Betaproteobacteria,* might be a process capable of providing pregnant woman additional metabolic power or a consequence of the change in pregnant women themselves providing differences in growth conditions for the microbiota as discussed by Jandhyala et al[30] and Ferrocino et al[31].

The overall inference of antibiotic use, years living in Norway, and country of birth were all factors that could affect microbiota diversity and composition[32]. We addressed these topics in our analysis; however, they had no significant impact. Moreover, the wide range of characteristics included in our study, such as the different number of years spent in Norway and the many different countries of birth in our sample, strengthened our study. Despite the presence of these variables, we found a significant differences in faecal microbiota (both in diversity and composition) between hyperemesis patients and healthy pregnant women. We also examined the implication of carriage of *H. pylori* in the microbiota of healthy pregnant women. No definite differences depending on the presence of *H. pylori* have been observed concerning the general findings referred to earlier[3].

Our observations are intriguing as we were aware that gut flora changes during pregnancy in the second and third trimesters[5]. A previous study using the same dataset showed 72.6% of women with hyperemesis had gestational age of < 12 weeks. By contrast, 94.4% controls had gestational age of > 12 weeks[23]. Cases were included during admittance to hospital due to hyperemesis during first and second trimesters. By contrast, faeces from controls were collected during the second trimester as the pregnant women were asked to participate at routine ultrasound screening thereafter sending faeces specimens *via* ordinary mail. Regardless of these differences, hyperemesis patients had a greater diversity of taxa present in contrast to what is normally seen during pregnancy[7].

We recognise that the handling of the material could have been more optimal, as materials from pregnant women being seen on an outpatient basis were initially sent by mail to the laboratory, thus being subject to variation in temperature in contrast to the material from hospitalised individuals, which were readily put in a freezer. This is a weakness of the study. One might speculate that the less sophisticated handling of the specimens from the non-hyperemesis group of patients might provide a greater diversity of microbiota. More likely, the abundance of microbes might increase in the non-hyperemesis group without new taxa necessarily turning up. Thus, with these limitations in mind, we present our findings as we believe they are relevant. In support of our handling procedure, several studies[33-35] have suggested that the implication of variations in handling specimens for later examination did not have a severe effect on results obtained later.

In short, there was an apparent further progression in clustering as seen in the second and third trimester of healthy pregnant; however, more and varied taxa appeared. This implies that the observations reported on gut flora in pregnant women[8,25] deviates in hyperemesis patients. A certain cluster of taxa with a higher capacity for survival in the changed environment might be a valid speculation. Even so, we believe that this serious diagnosis in pregnancy merits further studies, including studies on the possible interaction between human microbiota and pregnant women.

CONCLUSIONS

In conclusion, our results suggest a difference between women with hyperemesis and controls, with women with hyperemesis displaying more clustered alpha diversity with higher average numbers of different OTUs. The present data suggest that diversity in faecal microbiota in women with hyperemesis is higher than women who do not have hyperemesis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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