

## Gut microbiome patterns depending on children's psychosocial stress: Reports versus biomarkers

Nathalie Michels<sup>a,\*,1</sup>, Tom Van de Wiele<sup>b</sup>, Fiona Fouhy<sup>c,d</sup>, Siobhain O'Mahony<sup>d,e</sup>, Gerard Clarke<sup>d,f</sup>, James Keane<sup>d</sup>

<sup>a</sup> Department of Public Health, Faculty of Medicine and Health Sciences, Ghent University, Belgium

<sup>b</sup> Laboratory of Microbial Ecology and Technology, Faculty of Bio-engineering, Ghent University, Ghent, Belgium

<sup>c</sup> Teagasc Food Research Centre, Cork, Ireland

<sup>d</sup> APC Microbiome Ireland, University College Cork, Cork, Ireland

<sup>e</sup> Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

<sup>f</sup> Department of Psychiatry and Neurobehavioral Science, University College Cork, Cork, Ireland

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### ABSTRACT

**Aim:** Chronic stress increases disease vulnerability factors including inflammation, a pathological characteristic potentially regulated by the gut microbiota. We checked the association between the gut microbiome and psychosocial stress in children/adolescents and investigated which stress parameter (negative versus positive emotion, self-report versus parental report, events versus emotions, biomarker cortisol versus parasympathetic activity) is the most relevant indicator herein.

**Methods:** Gut microbiome sequencing was completed in fecal samples from 93 Belgian 8-16y olds. Stress measures included negative events, negative emotions, emotional problems reported by parents, happiness, hair cortisol and heart rate variability (pnn50 parameter reflecting parasympathetic activity). Alpha diversity, beta diversity and linear discriminant analysis were the unadjusted analyses. Age, sex, socio-economic status, diet, physical activity, sleep and weight status were adjusted for via a redundancy analysis and differential abundance via zero-inflated negative binomial regression.

**Results:** High stress as reflected by low pnn50 and more negative events were associated with a lower alpha diversity as indicated by the Simpson index. Happiness and pnn50 showed significant differences between high and low stress groups based on weighted UniFrac distance, and this remained significant after confounder adjustment. Adjusted and unadjusted taxonomic differences were also most pronounced for happiness and pnn50 being associated respectively with 24 OTU (= 11.8% of bacterial counts) and 31 OTU (= 13.0%). As a general pattern, high stress was associated with lower Firmicutes at the phylum level and higher *Bacteroides*, *Parabacteroides*, *Rhodococcus*, *Methanobrevibacter* and *Roseburia* but lower *Phascolarctobacterium* at genus level. Several genera gave conflicting results between different stress measures e.g. *Ruminococcaceae UCG014*, *Tenericutes*, *Eubacterium coprostanoligenes*, *Prevotella 9* and *Christensenellaceae R7*. Differential results in pre-adolescents versus adolescents were also evident.

**Conclusion:** Even in this young healthy population, stress parameters were cross-sectionally associated with gut microbial composition but this relationship was instrument specific. Positive emotions and parasympathetic activity appeared the strongest parameters and should be integrated in future microbiota projects amongst other stress measures.

### 1. Introduction

The body's goal in acute stress situations is to maintain stability through changes in the nervous and endocrine system for an

appropriate amount of time, but also to turn off these reactions immediately afterwards. In contrast, chronic stress leads to prolonged activation or inefficient management of these systems, with detrimental physiological consequences (McEwen, 1998, 2007). Consequently,

\* Corresponding author.

E-mail address: [Nathalie.michels@ugent.be](mailto:Nathalie.michels@ugent.be) (N. Michels).

<sup>1</sup> 4K3 De Pintelaan 185, 9000 Gent, Belgium.

chronic stress increases vulnerability to diseases like cardiovascular pathology (Ghike, 2016; Rohleder, 2014; Rosmond, 2005; Wirtz and von Kanel, 2017), even during childhood this associations is already present (Berens et al., 2017; Pervanidou and Chrousos, 2012). The underlying complex processes and mechanisms are still poorly understood but can help in designing prevention and treatment strategies. Apart from subjective reports, biological stress measures are necessary in this type of research because the nature and the chronicity of the stressor, as well as the individual's vulnerability and stress perception, are important in determining the physiological stress response and thus the adverse effects of chronic stress (Miller et al., 2007). Two main physiological stress systems exist (Charmandari et al., 2005). The first stress system is the hypothalamic-pituitary-adrenal (HPA) axis with cortisol as the end product. The second stress system is the autonomic nervous system with the catecholamines adrenaline and noradrenaline as end products. Heart rate variability (HRV) is often used as a non-invasive biomarker to indirectly measure cardiac parasympathetic and sympathetic activity (Task Force of ESC/NASPE, 1996).

In the stress-disease link, a possible explanation and intervention target might be the gut microbiota since there is bidirectional communication between the gut microbiota and the brain, (Aroniadis et al., 2017; Grenham et al., 2011; Moloney et al., 2014; Wang and Kasper, 2014) and the gut microbiome has been associated with metabolic syndrome (de Clercq et al., 2017). This relationship is sometimes called the 'microbiota-gut-brain axis' as the gut microbiota play an active role in the gut-brain communication. This microbiota-gut-brain axis consists of neural (autonomic and enteric nervous system), neuro-immune and neuro-endocrine (gut-epithelial enterochromaffin cells, the cortisol axis and bacterial-produced neuroactive molecules) components. Instability (compositional flux, rapid changes, easy disruption) and immaturity (low diversity, certain taxa not yet present) of the gut microbiota during childhood and adolescence increases the susceptibility to environmental insults such as stress and poor diet, which could result in gut dysbiosis and a deterioration in physical and mental health (Borre et al., 2014). The gut microbiota thus represents a potential therapeutic target.

The impact of stress on the gut microbiota is a topic on intense research scrutiny. Although several articles have been published on clinical depression cases (Jiang et al., 2015; Kelly et al., 2016; Lin et al., 2017; Naseribafrouei et al., 2014; Zheng et al., 2016), the relationship with gut bacteria is quite conflicting e.g. alpha diversity is sometimes decreased, sometimes non-significant and occasionally increased. On the other hand, gut microbiota transplantation from patients with depression into rodents successfully induced a depressive phenotype in these animals, demonstrating the powerful influence the gut microbiota can exert on behavior (Kelly et al., 2016; Zheng et al., 2016). A meta-analysis has shown an overall improvement of psychological outcomes in healthy humans by probiotics, i.e. by supplementation with health-beneficial bacteria (McKean et al., 2017). Observational research in healthy participants is scarce and often with limited results: reduced fecal lactic acid bacteria during exam periods (Knowles et al., 2008), no associations at all of gut microbiome with depressive symptoms or perceived stress (Kleiman et al., 2017), less emotional arousal with higher fecal *Prevotella* abundance (Tillisch et al., 2017) and fecal genera differences depending on mood but no clear patterns for depressed, anxious or angry mood (Li et al., 2016). Therefore, our goal was to see whether this link from stress/psychiatric symptoms to alterations in gut microbial composition/function translates to subthreshold psychological variation in healthy individuals i.e. children/adolescents. After all, childhood is an important period during which host-microbiome interactions establish appropriate stress responses. Indeed, work from Sudo and colleagues illustrated that there are critical time windows for gut microbiota assembly during early life, outside of which aberrant phenotypes may not be amenable to rescue (Sudo et al., 2004).

Indeed, both the main biological stress axes i.e. the HPA axis (Farzi et al., 2018) and the nervus vagus (Bonaz et al., 2018) system are

important components of the gut-brain axis, the framework through which the gut microbiota communicates with the central nervous system. For example, probiotic supplementation provoked in rats vagal afferent nerve impulses and suppressed stress-induced cortisol activation (Takada et al., 2016). Nevertheless, stress biomarkers are seldom applied in the observational human microbiome studies, with the parasympathetic activity in particular being under-researched. Therefore, we wanted to check how well stress biomarkers, including cortisol and autonomic nervous system markers, behave compared to questionnaires when studying the gut microbiota.

Taken together, we want to cover two neglected aspects of stress-microbiota research. Our first general aim was to check whether the cross-sectional association between gut microbiome and psychosocial stress already exists in children/adolescents. Secondly, we wanted to detect which reports (events versus emotions; positive versus negative emotions; parental versus self-report) and which biomarkers (cortisol versus HRV) are the most relevant indicator of these interactions.

## 2. Methodology

### 2.1. Study participants

Participants were Dutch-speaking Belgian children recruited for the longitudinal ChiBS study (Michels et al., 2012). Inclusion criteria at that moment were living in the region Aalter and age. During March-May 2015, an extra study wave took place when the participants were between 8 and 16 years old. At that time, a fecal sample was collected and stress was measured by hair cortisol analysis, HRV analysis and questionnaires (negative events, negative emotions, emotional problems and happiness). None of the participants had a reported diagnosis of Cushing/Addison disease, auto-immune disease, heart disease, chronic bowel disease or acute infection. Nobody took oral anti-inflammatory drugs or selective serotonin reuptake inhibitors in the 3 months before sample collection. One participant using oral corticosteroids and one participant using antibiotics in the previous 3 months were excluded. No information was available on psychiatric diagnosis or intervention. From the 242 participants, 104 collected a valid fecal sample for sequencing (optional module). After excluding those with low quality sequencing ( $n = 11$ ), 93 remained. All these participants had complete data on questionnaires but HRV data was missing in 7 of them and hair cortisol data was missing in 28. Thus, only 61 had full data on both biomarkers. Those with biomarker info had more emotional problems than those without biomarker info but did not differ in the tested confounders. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and the project protocol was approved by the Ethics Committee of the Ghent University Hospital. A written informed consent was obtained from the parents and a verbal assent from the minors.

### 2.2. Fecal samples

Participants received a flushable toilet seat cover for stool collection (Fe-Col®, Col-group, Amsterdam, The Netherlands) and a plastic screw cap container with spoon. In this way, fecal collection occurred in a participant-friendly manner without contamination by toilet water. The participants were asked to store the samples immediately at  $-20^{\circ}\text{C}$  and after at-home collection (as soon as possible, on average within one week) samples were transferred on ice to the laboratory for further storage at  $-80^{\circ}\text{C}$ .

#### 2.2.1. DNA extraction

Bacterial DNA was extracted as described earlier (Vilchez-Vargas et al., 2013), using a Lysis Buffer (TrisEDTA, NaCl, PVP40, SDS, water) and glass beads for FastPrep. Extraction was performed with phenol-chloroform and EtOH/NaOAc was used for precipitation (Boon et al., 2003). Samples were dissolved in TrisEDTA 1X and stored at  $-20^{\circ}\text{C}$ .

Concentration and quality were verified by Glomax Multi Detection system (Promega, USA) and 2% agarose gel electrophoresis.

### 2.2.2. Polymerase chain reaction

All samples were prepared starting from 1:100 dilutions. Using a thermocycler (2720 Thermal cycler, Applied Biosystem, Foster City, USA), DNA was denaturated for 5 min at 94 °C and then 30 cycles of denaturation 1 min at 95 °C, hybridization for 1 min at 53 °C, elongation for 2 min at 72 °C were run, to end with a final elongation of 10 min at 72 °C.

### 2.2.3. Illumina sequencing

Samples were analyzed by LGC Genomics (Berlin, Germany) on an Illumina Miseq Platform for the V3-V4 region 16S rDNA. Three hundred base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). 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 was completed using QIIME (version Version 1.9.0). Denoising, chimera detection and clustering into operational taxonomic unit (OTU) grouping were performed using USEARCH v7 (64-bit) (Edgar, 2010). OTUs were aligned using PyNAST (PyNAST: a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release 123. Samples with less than 10,000 OUT counts were excluded. Joining efficiency was around 90% and only 0.9% chimera's were found. After removal of short reads, chimera's and singletons, a mean of 24,655 OTU-linked sequences/sample was obtained (SEM = 1121; maximum was 66512). No rarefaction was executed; 1039 different OTUs were included in the analyses.

### 2.3. Hair cortisol

Hair cortisol has recently been established as a reliable marker of chronic stress exposure (Wester and van Rossum, 2015). Only the most proximal 3 cm of the hair strands from the vertex posterior were analyzed. Since hair grows approximately 1 cm each month, this 3 cm reflects the exposure during the last 3 months. On 15 mg hair, extraction and liquid chromatography coupled with tandem mass spectrometry (AB Sciex 5500 triple-quadrupole) was performed at the Laboratory for Hormonology, Ghent University Hospital. Data processing was performed through MultiQuant version 2.0.2. Inter-assay CV for cortisol was 10.8% with a limit-of-quantification of 1.6 pg/mg hair.

### 2.4. Heart rate variability

To define HRV, each participant was individually examined in a quiet room in supine position (i.e. lying down with the face up) during 10 min. Participants were asked to refrain from strenuous physical activity on the measurement day. The participant was encouraged to be calm, to breath normally and not to speak or move during the HRV measurement. The heart rate belt was fixed around the chest and measurements were started after a couple of minutes when the signal was stabilized. RR-intervals were recorded at a sampling rate of 1000 Hz with the elastic electrode belt Polar Wear link 31 using a Wind link infrared computer transmitter. This low-cost device has a proven validity compared to the gold standard of an electrocardiogram device, also in children (Gamelin et al., 2008). Data processing was performed with the free, professional HRV Analysis Software of the University of Kuopio (Niskanen et al., 2004). The middle 5 min were manually checked on their quality and if necessary, another appropriate 5 min interval was chosen. The RR series were de-trended using the Smoothness priors method with  $\alpha = 300$  and a cubic interpolation at the default rate of 4 Hz was done. In the time domain analysis, pnn50 (percentage of successive normal sinus RR intervals > 50 ms) was used as marker of the parasympathetic activity.

### 2.5. Negative events

The Coddington Life Events Scale for Children (CLES-C) is a validated and well-established 36-item questionnaire (test-retest  $r = 0.69$ , parent-child agreement ICC = 0.45) (Coddington, 1999). By child self-report, it assesses the prevalence, frequency and timing of stressful life events relevant for this age group during the last year. By measuring significant life events in terms of Life Change Units depending on timing, frequency and severity, the questionnaire can provide insight into recent events that may affect the child's health. For the current analyses, only negative events were considered.

### 2.6. Negative and positive emotions reported by self-report

Participants had to report how they mostly feel (not only on the examination day). The feelings happy, anger, anxiety and sadness were rated on a 0 to 10 Likert-scale (0 'not at all' to 10 'very strong'). The sum of anger, anxiety and sadness was used to represent 'negative emotions'. We have validated this parameter with the PANAS-C questionnaire (Laurent et al., 1999) in a sample of 153 9–12 years old children: our negative emotions score showed a Spearman correlation of  $r = 0.48$  ( $p < 0.001$ ) with the negative affect score of the PANAS-C.

### 2.7. Emotional problems reported by the parent

Parents were asked to complete the standardized 'Strengths and Difficulties Questionnaire' for their child (Goodman, 1997) (Cronbach's  $\alpha = 0.53$ –0.76, test-retest stability  $r = 0.88$ , concurrent validity  $r = 0.7$ –0.87). The subscale on emotional problems over the past 6 months (proxy-report) with 5 items was used.

### 2.8. Potential confounders

Age was calculated and gender reported. To represent socio-economic status, parental education level was assessed by questionnaire according to the International Standard Classification of Education. BMI was calculated by dividing measured weight with height squared ( $\text{kg}/\text{m}^2$ ). The Flemish growth reference data of 2004 were used to compute the z-score of BMI (zBMI) to adjust for age and gender (Cole and Lobstein, 2012). Based on a food-frequency questionnaire (Lanfer et al., 2011), intake frequency of fiber-rich food, protein-rich food, sweet food and fatty food were calculated. Daily sleep duration was calculated from weighted weekday and weekend day sleep duration:  $\text{weekday} * 5 + \text{weekend day} * 2/7$ . Mean physical activity per day was calculated from reported active time outside and sport club hours.

### 2.9. Statistical analyses

Descriptive statistics were retrieved in SPSS using Spearman's rank correlation coefficient. Median, 25th percentile and 75th percentiles were also reported. Statistical significance was considered at a  $P < 0.05$  level of confidence after False Discovery Rate correction except for the explorative correlation matrix. For every analysis, the six stress parameters were considered (negative events, negative emotions, emotional problems, happiness, pnn50, cortisol). All stress data was continuous but was dichotomized where necessary for some analyses: tertiles were used to create a 2/3th subsample with low stress versus 1/3th with high stress. To detect developmental differences, analyses were also repeated for preadolescents (< 12y,  $n = 64$ ) and adolescents ( $\geq 12y$ ,  $n = 47$ ).

Alpha diversity/richness indices (observed species, Chao1 and Simpson diversity) were calculated in QIIME (Caporaso et al., 2010) and then used as outcomes in linear regression adjusted for age, gender, parental education, zBMI, protein, fiber, sweet, fat intake, physical activity and sleep. For beta diversity, principal coordinates analysis (PCoA) was performed using unweighted and weighted UniFrac

**Table 1**  
Descriptive data by median, interquartile range and Spearman correlation coefficients (n = 93).

	Median [p25;p75]	Negative events	Negative emotions	Emotional problems	Happy	Pmn50	Hair cortisol	Age	Bmi z-score	Fiber intake	Protein intake	Fatty food intake	Sweet food intake	Physical activity	Sleep	Gender	Parental education
<b>Stress data</b>																	
Negative events [score]	47 [0;104]																
Negative emotions [0–10]	5 [3;7]	0.008															
Emotional problems [0–10]	2 [1;3]	0.047	0.273**														
Happy [0–10]	9 [8;10]	0.024	-0.186*	-0.059													
pmn50 [%] (n = 65)	42 [29;59]	-0.048	-0.107	-0.103	0.220*												
Hair cortisol [pg/mg] (n = 86)	3.7 [2.8;5.1]	-0.057	0.099	0.018	0.103	0.047											
<b>Confounder data</b>																	
Age [years]	12 [10.8;13.6]	0.100	0.273**	0.034	-0.015	-0.171	0.137										
Bmi z-score	-0.28 [-0.74;0.25]	0.032	0.155	-0.092	-0.190*	-0.134	-0.057	0.067									
Fiber-rich food intake [times/week]	30.2 [20.9;42.25]	-0.071	-0.100	-0.035	0.185	0.028	0.016	0.036	-0.111								
Protein-rich food intake [times/week]	30.8 [22.4;39.5]	0.114	-0.266**	-0.219*	-0.004	0.114	0.025	0.051	0.080	0.318**							
Fatty food intake [times/week]	26.3 [18.5;33.9]	0.076	-0.018	0.066	-0.131	0.076	-0.178	0.085	0.073	0.186	0.396**						
Sweet food intake [times/week]	29.3 [21.3;37.9]	-0.092	-0.130	0.137	-0.022	0.205	-0.399**	-0.128	-0.054	0.424*	0.263*	0.564**					
Physical activity [hours/day]	1.1 [0.9;1.9]	0.133	-0.032	-0.177	0.134	0.085	-0.013	0.030	-0.104	0.007	0.047	0.083	0.024		0.019		
Sleep [hours/day]	9.6 [9;10.3]	-0.139	-0.223*	-0.073	0.139	0.218*	0.018	-0.605**	-0.189	0.020	-0.001	-0.152	-0.012	-0.142			
Gender [% girls]	48%	0.107	0.190	0.272**	-0.127	-0.091	0.054	-0.022	-0.002	-0.057	-0.166	0.044	0.085	-0.122	-0.050		
Parental education [% post-secondary]	82.9%	-0.078	-0.074	-0.119	0.126	0.063	-0.017	-0.103	0.039	0.261*	0.074	0.097	0.150	0.017	0.121	0.019	
<b>Bacterial data</b>																	
Euryarchaeota [%]	0 [0;0.7]	0.135	-0.002	0.185	-0.243*	-0.051	-0.182	0.067	0.084	-0.159	-0.147	0.029	-0.096	-0.117	-0.204*	0.078	-0.267**
Actinobacteria [%]	2.8 [1.2;5.4]	-0.079	-0.112	-0.010	0.004	0.022	0.015	-0.088	-0.014	-0.116	0.014	0.065	0.070	-0.264*	-0.087	0.142	0.031
Bacteroidetes [%]	12.3 [4.7;23.3]	-0.044	0.052	-0.083	-0.202	-0.200	0.077	0.095	0.118	-0.154	-0.010	0.030	-0.085	-0.070	-0.042	0.072	-0.268**
Cyanobacteria [%]	0 [0;0.003]	0.014	-0.147	0.118	0.209*	0.135	-0.205	-0.013	-0.151	0.125	0.077	0.047	0.160	0.031	-0.058	0.014	0.124
Firmicutes [%]	75.1 [63.9;84.3]	0.036	-0.074	-0.029	0.314*	0.348**	0.072	-0.103	-0.048	0.087	-0.054	-0.037	0.019	0.139	0.106	-0.105	0.222*
Fusobacteria [%]	0 [0;0.002]	0.059	0.081	-0.044	-0.119	0.034	-0.176	-0.130	0.118	-0.096	-0.050	0.089	0.094	-0.071	-0.149	0.200	0.016
Proteobacteria [%]	0.19 [0.07;0.39]	-0.004	0.068	0.023	-0.219*	-0.168	0.228	0.204	-0.004	-0.303**	-0.136	-0.117	-0.260*	-0.179	-0.225*	0.162	-0.169
Saccharibacteria [%]	0.02 [0.01;0.06]	0.083	0.090	0.150	-0.043	-0.118	-0.044	0.145	0.034	-0.186	-0.070	0.030	-0.025	-0.086	-0.145	0.194	-0.081
Spirochaetae [%]	0 [0;0]	0.291**	0.127	0.025	-0.049	0.028	-0.176	0.138	0.027	0.190	-0.024	0.214*	0.018	0.047	-0.312**	-0.070	0.016
Tenericutes [%]	0.019 [0.006;0.939]	0.063	-0.158	0.158	-0.015	0.103	-0.139	-0.222*	-0.105	0.151	-0.078	-0.009	0.299**	0.015	0.101	0.072	0.050

(continued on next page)

Table 1 (continued)

	Median [p25;p75]	Negative events	Negative emotions	Emotional problems	Happy	Pnn50	Hair cortisol	Age	Bmi z-score	Fiber intake	Protein intake	Fatty food intake	Sweet food intake	Physical activity	Sleep	Gender	Parental education
Verrucomicrobia [%]	0.790 [0.008;4.757]	-0.037	-0.089	0.166	0.017	0.020	-0.068	-0.007	-0.186	0.320**	-0.010	0.039	0.104	0.125	-0.012	-0.005	0.000
Firmicutes/Bacteroidetes [%]	6.3 [2.9;16.1]	0.046	-0.040	0.074	0.223*	0.213*	-0.060	-0.108	-0.120	0.144	-0.013	-0.040	0.065	0.085	0.061	-0.089	0.277**
Observed species	331 [293;361]	-0.135	0.085	0.087	-0.084	0.158	-0.146	-0.064	-0.122	0.115	0.125	0.143	0.268**	-0.102	0.052	0.102	0.169
Chao1 richness	396 [353;445]	-0.079	0.033	0.123	-0.174	0.112	-0.167	-0.131	-0.161	0.119	0.168	-0.053	-0.006	0.015	-0.047	0.122	0.127
Simpson diversity [0-1]	0.966 [0.953;0.974]	-0.153	0.123	0.062	0.212*	0.178	-0.102	0.047	0.012	-0.066	0.131	0.103	0.210*	-0.091	0.000	-0.169	0.041

\* p < 0.05, \*\* p < 0.01.

distances and significant differences between stress groups were determined by permutational multivariate analysis of variance using the vegan package in R (Oksanen et al., 2018). Taking the phylogenetic framework of microbial communities into account, a distance based redundancy analysis was also carried out using weighted UniFrac distances as described in (Shankar et al., 2017). This constrained form of ordination assesses which of the continuous stress variables are driving the changes seen in microbial communities between samples while partialling out the variation from confounding variables.

Taxonomic features distinguishing between high and low stress groups were identified using the linear discriminant analysis (LDA) effect size (LefSe) method for biomarker discovery, which emphasizes both statistical significance and biological relevance (metagenomic biomarker discovery and explanation). LefSe uses the Kruskal–Wallis rank-sum test with a normalized relative abundance matrix to detect features with significantly different abundances between assigned taxa and performs LDA to estimate the effect size of each feature (Segata et al., 2011). Cladograms (implemented using the Galaxy framework at <http://huttenhower.sph.harvard.edu/lefse/>) are shown for effect size > 2.0. As this technique does not allow to adjust for confounders, we also assessed the differential distributions of microbial communities based on a zero-inflated negative binomial (ZINB) regression model as outlined by (Chen et al., 2018a). Differences in distributions between low and high stress groups with adjusted p-value < 0.1 were found based on a chi squared statistic that takes into account both the abundance (mean) and dispersion (SD) of OTU counts as well as prevalence of zeros in the data, while adjusting for the distributions of confounders.

### 3. Results

#### 3.1. Descriptive data

Table 1 summarizes descriptive data and the Spearman correlation coefficients for stress variables, confounders, bacterial phyla and alpha-diversity. From the stress reports, negative emotions (self-report) and emotional problems (parental report) were positively significantly related to each other and happiness was negatively related with negative emotions. The two stress biomarkers were not related to each other, only pnn50 was positively related to happiness and borderline negatively to some other reports. In these 93 participants, only 3.2% were overweight. Based on questionnaire specific cut-offs, 31% were at risk for stress by experienced events and 24.7% were at risk for stress based on their emotional report. Most abundant families were Rumino-coccaceae (43%; most frequent genus *Faecalibacterium*), Lachnospiraceae (15%, most frequent genus *Pseudobutyrvibrio*) and Bacteroidaceae (4%; most frequent genus *Bacteroides*). Top 10 abundant OTU represented together 25.6% of all OTU counts and were in descending order *Ruminococcus2* for 3.7%, *Dialister*, *Faecalibacterium*, *Faecalibacterium*, *Subdoligranulum*, *Pseudobutyrvibrio*, *Dialister*, *Ruminococcaeae UCG002*, *Pseudobutyrvibrio* and *Ruminococcus2*.

#### 3.2. Alpha diversity

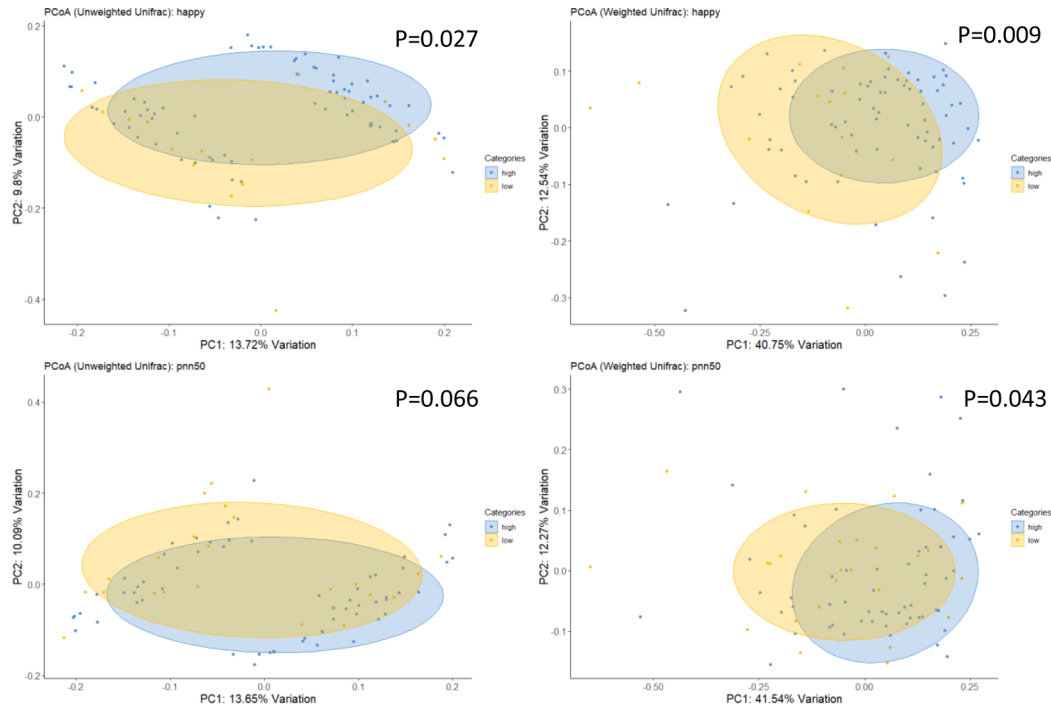
The relation of stress measures with alpha diversity is shown in Table 2. High stress as reflected by more negative events and low pnn50 was significantly related to lower diversity as indicated by the Simpson index. In preadolescents after splitting by age, the same observations stayed significant but with additionally negative events being also related to less observed species (p = 0.015; mean low 324 vs high 256) and Chao1 (p = 0.03; mean low 394 vs high 332) and negative emotions being related to more observed species (p = 0.006, mean low 290 vs high 368). On the other hand, no significant differences in alpha diversity by stress were found in the adolescent sample.



**Table 2**  
Alpha diversity for the six stress parameters.

	negative events			negative emotions self-report			emotional problems parental report			happy			pnn50			hair cortisol		
	low	high	p	low	high	p	low	High	p	low	high	p	Low	high	p	low	high	p
Observed species	325	295	0.120	305	336	0.103	311	319	0.852	329	308	0.312	300	320	0.326	323	312	0.754
Chao1	394	372	0.351	376	412	0.092	382	392	0.814	416	375	0.187	373	392	0.435	394	381	0.801
Simpson diversity	0.964	0.949	0.022*	0.957	0.962	0.403	0.955	0.966	0.116	0.950	0.962	0.201	0.948	0.964	0.024*	0.960	0.962	0.644

Based on linear regression adjusted for age, gender, parental education, zBMI, fiber, protein, sweet food, fatty food intake, physical activity and sleep.



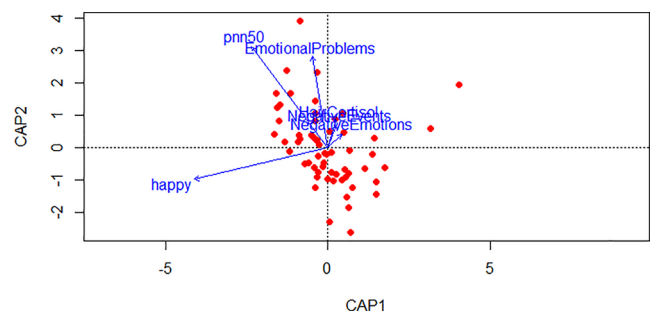
**Fig. 1.** Weighted and unweighted Unifrac Principal Coordinates Analysis for pnn50 and happy.

**3.3. Beta diversity**

Weighted and unweighted Unifrac PCoA was carried out. Only for pnn50 and happiness a significant separation between high and low stress groups was found (see Fig. 1). The plots for the four other stress measures and for the two separate age-groups can be found in Appendix A. When separated by age-group, happiness and pnn50 still showed significant microbiome separation in preadolescents but not in adolescents. In agreement with this finding, dbRDA (Fig. 2) also found that especially happiness followed by pnn50 contributed significantly to the variance in microbial communities between samples. Split by age group, happiness contributed significantly to microbial variety in preadolescents ( $p = 0.031$ ), while pnn50 contributed only borderline in adolescents ( $p = 0.074$ ).

**3.4. Phylogenetic differences**

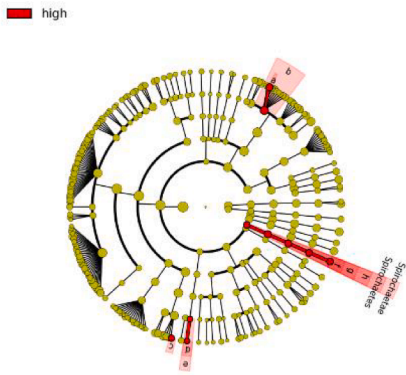
To detect which taxonomic differences distinguished stress levels, LefSe was executed. As can be seen in Fig. 3, again pnn50 and happy gave the clearest distinction in microbiota composition and also had several mutual distinctive taxa. On phylum level, low pnn50 or happiness and thus high stress was associated with a lower Firmicutes relative abundance, mainly the order Clostridiales (most often genera under the family of Lachnospiraceae and Ruminococcaceae). The phylum Bacteroidetes abundance was also associated with low



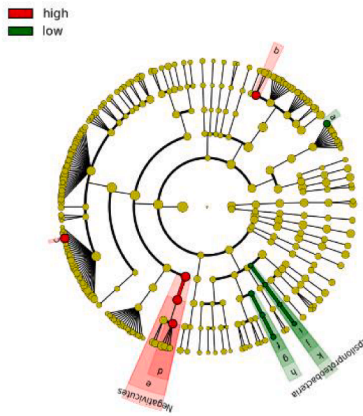
**Fig. 2.** Unifrac distance based redundancy analysis plot for the six stress measures. Biplot of Weighted Unifrac distance based redundancy analysis where each red dot represents a sample and vectors represent the continuous stress measures each pointing to the direction to which they exhibit the strongest association while their magnitude indicates the strength of the variable in explaining the dispersion observed. The variance contributed by age, zBMI, gender, parental education, diet, physical activity and sleep has been partialled out. Associations were significant for pnn50 and happiness only.

happiness, mainly the order Bacteroidales. The only other significant phylum was the Euryarchaeota, with higher *Methanobrevibacter* in the low happiness group. Differential taxa for the other stress parameters had less consistency or overlap.

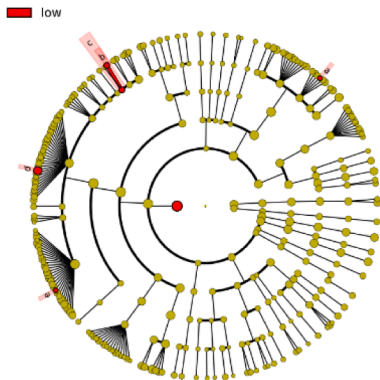
Negative events



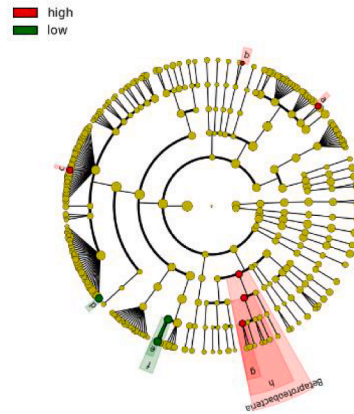
Negative emotions



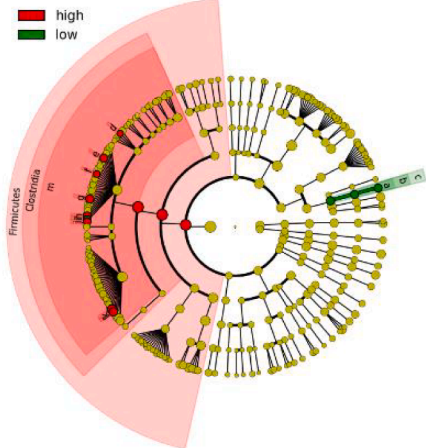
Emotional problems



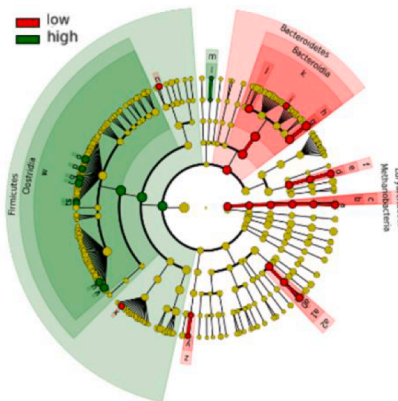
Hair cortisol



pnn50



happy



**Fig. 3.** Linear discriminant analysis effect size (LEfSe) cladogram for the six stress parameters. **Negative events:** a) *Prevotellaceae UCG001*, b) *Prevotellaceae*, c) *Veillonellaceae uncultured*, d) *Sneathia*, e) *Leptotrichiaceae*, f) *Brachyspira*, g) *Brachyspiraceae*, h) *Spirochaetales*. **Negative emotions:** a) *Coriobacteriaceae uncultured*, b) *Rikenellaceae*, c) *Faecalibacterium*, d) *Veillonellaceae*, e) *Selenomonadales*, f) *Methylophilaceae uncultured*, g) *Methylophilales*, h) *Methylophilales*, i) *Sulfurovum*, j) *Helicobacteraceae*, k) *Campylobacteriales*. **Emotional problems:** a) *Coproacter*, b) *Clostridiales Vadin BB60 uncultured*, c) *Clostridiales Vadin BB60*, d) *Pseudobutyrvibrio*, e) *Ruminococcaceae UCG*. **Hair cortisol:** a) *Bacteroidales S24-7 uncultured*, b) *Sediminibacterium*, c) *Lachnospiraceae UCG005*, d) *Ruminococcaceae uncultured*, e) *Phascolarctobacterium*, f) *Acidaminococcaceae*, g) *Alcaligenaceae*, h) *Burkholderiales*. **pnn50:** a) *Rhodococcus*, b) *Nocardiaceae*, c) *Corynebacteriales*, d) *Family XIII UCG001*, e) *Coprococcus2*, f) *Lachnospira*, g) *Roseburia*, h) *Eubacterium ventriosum*, i) *Ruminococcus gaurvreauii*, j) *Lachnospiraceae uncultured*, k) *Ruminococcaceae UCG014*, l) *Ruminococcus1*, m) *Clostridiales*. **Happy:** a) *Methanobrevibacter*, b) *Methanobacteriaceae*, c) *Methanobacteriales*, d) *Rhodococcus*, e) *Nocardiaceae*, f) *Corynebacteriales*, g) *Bacteroides*, h) *Bacteroidaceae*, i) *Porphyromonas*, j) *Rikenellaceae*, k) *Bacteroidales*, l) *unclassified*, m) *unclassified*, n) *Lactococcus*, o) *Lachnoclostridium*, p) *Lachnospiraceae NK4A136*, q) *Lachnospiraceae UCG008*, r) *Roseburia*, s) *Ruminococcus gaurvreauii*, t) *Lachnospiraceae uncultured*, u) *Ruminococcus1*, v) *Eubacterium coprostanoligenes group*, w) *Clostridiales*, x) *Coproacillus*, y) *Sneathia*, z) *Leptotrichiaceae*, a0) *Escherichia-Shigella*, a1) *Enterobacteriaceae*, a2) *Enterobacteriales*.

When separating by age group (see Appendix B), very often different stress-differentiating taxa appeared for preadolescents versus adolescents. For happiness and pnn50, there were more differentiating taxa in preadolescents than in adolescents, while the opposite was true for the other stress parameters. On phylum level, lower Firmicutes with higher stress was found in both age groups, while lower Bacteroidetes with higher pnn50 (i.e. low stress) was only present in adolescents, and the association between happiness and lower Proteobacteria was only true in preadolescents.

Using differential abundances by zero-inflated binomial regression based on OTU's and adjusted for confounders (see Table 3 for summarized data and Appendix C for full data), several of these findings were confirmed. Effect sizes in terms of Cohen's d ranged from small (< 0.2) to very high (maximum observed = 1.8) but mostly the difference in specific OTU between high and low stress was around a half to one standard deviation. The amount of significant OTU's differed per stress parameter: 1 OTU (=0.8% of all counts) for hair cortisol, 16 OTU

**Table 3**  
Significant differential abundances by zero-inflated binomial regression for the six stress parameters.

	Negative events	Negative emotions	Emotional problems	Low happiness	Hair cortisol	Low pnn50
Actinobacteria Bifidobacteriaceae <i>Bifidobacterium</i>		+		+		-
Actinobacteria Coriobacteriaceae <i>Enterorhabdus</i>			+/-			-
Actinobacteria Nocardiaceae <i>Rhodococcus</i>				+		+
Bacteroidetes Bacteroidaceae <i>Bacteroides</i>	+	+	+	+		+
Bacteroidetes Porphyromonadaceae <i>Barnesiella</i>				+		
Bacteroidetes Porphyromonadaceae <i>Parabacteroides</i>		+		+		+
Bacteroidetes Prevotellaceae <i>Prevotella 9</i>		+/-	-	+/-		-
Bacteroidetes Rikenellaceae <i>Alistipes</i>			+			
Euryarchaeota Methanobacteriaceae <i>Methanobrevibacter</i>				+		+
Firmicutes Acidaminococcaceae <i>Phascolarctobacterium</i>		-	-			-
Firmicutes Christensenellaceae <i>Christensenellaceae R-7 group</i>	-	-	-	-		+
Firmicutes Erysipelotrichaceae <i>Holdemania</i>			+			-
Firmicutes Lachnospiraceae <i>Ruminantium</i>	+		-			
Firmicutes Lachnospiraceae <i>Blautia</i>			+	-		-
Firmicutes Lachnospiraceae <i>Coprococcus 1</i>						+
Firmicutes Lachnospiraceae <i>Coprococcus 2</i>	-					
Firmicutes Lachnospiraceae <i>Lachnoclostridium</i>				-		
Firmicutes Lachnospiraceae <i>Lachnospiraceae NK4A136 group</i>				-		-
Firmicutes Lachnospiraceae <i>Lachnospiraceae UCG-004</i>				-		
Firmicutes Lachnospiraceae <i>Roseburia</i>		+		+		
Firmicutes Lachnospiraceae <i>uncultured</i>						-
Firmicutes Ruminococcaceae [ <i>Eubacterium</i> ] <i>coprostanoligenes group</i>	+/-		+	+		+/-
Firmicutes Ruminococcaceae <i>Anaerotruncus</i>	+		-			+
Firmicutes Ruminococcaceae <i>Ruminiclostridium 5</i>			+			
Firmicutes Ruminococcaceae <i>Ruminococcaceae UCG002</i>						-
Firmicutes Ruminococcaceae <i>Ruminococcaceae UCG003</i>						-
Firmicutes Ruminococcaceae <i>Ruminococcaceae UCG004</i>		+				
Firmicutes Ruminococcaceae <i>Ruminococcaceae UCG010</i>		-	+			-
Firmicutes Ruminococcaceae <i>Ruminococcaceae UCG014</i>	+/-	-	+	+/-	+	+/-
Firmicutes Ruminococcaceae <i>Ruminococcus 2</i>				+		
Firmicutes Ruminococcaceae <i>uncultured</i>			-			
Firmicutes Veillonellaceae <i>Dialister</i>		-		+		+
Firmicutes Veillonellaceae <i>Veillonella</i>			-			
Tenericutes <i>uncultured</i>	+/-	-	-	+		-
Verrucomicrobia Verrucomicrobiaceae <i>Akkermansia</i>						-

+ : increased, - : decreased: +/- sometimes increased and sometimes decreased in the high stress group. Analyses were adjusted for age, gender, parental education, zBMI, fiber, protein, sweet and fat food intake, physical activity and sleep.

(= 4.7%) for negative events, 25 OTU (= 5.9%) for emotional problems, 15 OTU (= 6.2%) for negative emotions, 24 OTU (= 11.8%) for happy and 31 OTU (= 13.0%) for pnn50. Taken all stress measures together, 35 genera seemed distinctive towards stress. Genera with highest effect size were *Ruminococcaceae UCG014*, *Bacteroides*, *Phascolarctobacterium*, *Tenericutes uncultured*, *Eubacterium coprostanoligenes*, *Methobrevibacter* and *Blautia*. The most consistent finding was a positive association with *Bacteroides* for 5 of the 6 stress parameters, followed by three positive associations with *Parabacteroides*, three negative associations with *Phascolarctobacterium*, two negative associations with *Lachnospiraceae NK4A136* and two positive associations with *Rhodococcus*,

*Methanobrevibacter* and *Roseburia*. Significant OTU-stress associations for most stress parameters but in opposite directions (i.e. sometimes positive, sometimes negative) were found for *Ruminococcaceae UCG014*, *Tenericutes*, *Eubacterium coprostanoligenes*, *Prevotella 9* and *Christensenellaceae R7*. Overall, differential abundances depending on stress levels were seen within the phyla Actinobacteria, Bacteroidetes, Euryarchaeota, Firmicutes, Verrucomicrobia and Tenericutes. Results separated by age group can be found in [Appendix B](#). As a small sample size reduces performance of the zero-inflated binomial regression, only few significant taxa appeared in both children and adolescents.



## 4. Discussion

Our goal was to see whether the association between gut microbiota and stress is visible in the full spectrum instead of focusing on the extremes (i.e. those suffering from a psychiatric illness). Therefore, data was used from a group of 8–16y old children from the general population. More specifically, our goal was to detect which stress measures in the format of four questionnaires and two biomarkers would be the most sensitive in relation to gut microbiota composition. After all, [Table 1](#) showed that only a few of them are significantly interrelated and our previous research has shown that cortisol and HRV can reflect different stress exposures ([Michels et al., 2013](#)). This is the first study to consider the use of hair cortisol, HRV and positive emotions. For the first time, we show that a surrogate marker of the parasympathetic response to stress (i.e. pnn50) clearly corresponds to microbial composition. Similar findings were present for happiness. Indeed, pnn50 and happiness were positively correlated with each other (see [Table 1](#)). High stress as reflected by low pnn50 and more negative events was associated with lower alpha diversity by Simpson index. Pnn50 and happiness were found to significantly drive overall differences in microbiome variation between samples, based on weighted Unifrac PCoA and confounder adjusted RDA, thus independent of diet. Taxonomic differences based on LEfSe analyses were also most pronounced for pnn50, followed by happiness and negative emotions, while hair cortisol showed the least significant differences. Low pnn50 and thus high stress was associated with a decreased Firmicutes frequency. Based on differential distribution analysis of microbial communities, a consistent finding over at least 2 stress measures was the higher level of *Bacteroides*, *Parabacteroides*, *Rhodococcus*, *Methanobrevibacter* and *Roseburia* but lower *Phascolarctobacterium* and *Lachnospiraceae NK4A136*. Several genera gave conflicting results between different stress measures e.g. *Ruminococcaceae UCG014*, *Tenericutes*, *Eubacterium coprostanoligenes*, *Prevotella 9* and *Christensenellaceae R7*. Developmental issues seem important as the overall findings were more pronounced in preadolescents than adolescents and a lot of taxonomic differences existed between the two age-groups.

Most of the existing research has been done in rodent models, by selected probiotic intervention and as case-control studies in depression. Comparison with the existing literature should thus be done with caution. Studies on depression often include patients that take medication, have different severity/duration of depression or have comorbidities with inflammatory status while often no adjustments are done for confounders like diet. In our child/adolescent population, the stress measures were not consistently associated with inflammation (unpublished data) and participants taking medication were excluded. Moreover, subtle differences might exist in gut microbiome alterations as a consequence of stress-exposure versus as a consequence of clinical depression per se, although stress and depression share similar neurobiological pathways including HPA axis and autonomic dysfunction. Depression is a stress-related disorder and stress-induced depression-like behavior that manifests concurrently with stress-induced microbiota alterations is a feature of the preclinical literature. In rodents, the precise magnitude and direction of the microbiota shift may vary between different mouse strains and/or models of stress ([Bharwani et al., 2016](#)).

### 4.1. Alpha and beta diversity

We detected a decreased alpha diversity using the Simpson diversity matrix in stress reflected by negative events and pnn50. Although greater bacterial diversity is potentially beneficial to human health, its role in brain function remains subject to debate. In patients with depression, either no alterations in alpha diversity, sometimes less diversity and occasionally higher diversity have been reported ([Kuo and Chung, 2018](#)). In terms of beta diversity, conflicting results in literature have also been reported with either significant separations in humans

between groups or not ([Kuo and Chung, 2018](#)). In healthy women, no significant gut microbiome diversity differences depending on stress, anxiety or depression were found ([Kleiman et al., 2017](#)). In accordance with this, our negative emotions parameter did not show significant alpha or beta diversity. Nevertheless, significant separation of the stress groups was evident by PCoA plot and a clear association was seen using dbRDA for positive emotions. Indeed, positive and negative emotions have been shown to exhibit distinct effects on the human gut microbiota ([Li et al., 2016](#)). No studies have yet examined diversity based on cortisol or parasympathetic activity but we have now shown that parasympathetic activity (measured with pnn50) is a discriminator in the PCoA plot. The PCoA based on weighted Unifrac was more pronounced so it concerns rather shifts in relative abundance than the pure absence/presence of bacteria.

### 4.2. Overall taxonomic differences

At a phylum level we observed lower Firmicutes (mainly the order of Clostridiales) for stress as reflected by low pnn50 or low positive emotions, with then a counter reaction in Bacteroidetes (mainly the order of Bacteroidales) for positive emotions. Consequently, a lot of Lachnospiraceae and Ruminococcaceae OTU differences appeared in the more detailed analyses but with positive and negative associations. In general, a higher gut Firmicutes/Bacteroidetes ratio has been related with ill health like obesity, but this has recently been challenged ([John and Mullin, 2016](#)). In major depression, Firmicutes is more often underrepresented in fecal samples, while Bacteroidetes has a quite inconclusive trend of abundance ([Kuo and Chung, 2018](#)). In fact, research shows that disease is more than just the imbalance between phyla. Indeed, fecal OTU specific differences in e.g. Firmicutes have been detected with either increases or decreases in a social stress rodent model ([Bharwani et al., 2016](#)) and in a major depression group ([Zheng et al., 2016](#)).

Therefore, we looked further into the genus level. In major depression, fecal *Bacteroides* and *Blautia* are often overrepresented while *Bifidobacterium*, *Faecalibacterium* and *Dialister* are often underrepresented ([Kuo and Chung, 2018](#)). In addition, fecal *Alistipes*, *Prevotella* and *Roseburia* were reported to have opposite direction of abundance across human studies ([Kuo and Chung, 2018](#)). In healthy adults, fecal *Prevotella* was twice positively related to stress parameters ([Hantsoo et al., 2018](#); [Tillisch et al., 2017](#)). Thirty-five different genera appeared significant in our OTU-based regression. We limit the discussion towards our most consistent genera.

*Bacteroides*, the most frequent genus of the Bacteroidetes in our population, was increased for most stress parameters, while *Parabacteroides* was decreased. This is in agreement with the general findings on gut microbiota in human depression ([Kuo and Chung, 2018](#)) and healthy adults ([Li et al., 2016](#)). Related to this, fecal *Bacteroides* have been reproducibly associated with high-fat, high-protein Western-style diets, thus as a biomarker of unhealthy lifestyle. Another genus of this phylum, *Prevotella*, showed often inconsistent associations with four stress parameters and this inconsistent pattern is also mentioned in a review on human depression ([Kuo and Chung, 2018](#)). The other phylum with several stress associations was Firmicutes. Herein, *Ruminococcaceae UCG014* was the only genus associated with all six stress parameters but with mixed findings. In human depression, fecal Ruminococcaceae have been reported to be increased ([Zheng et al., 2016](#)) or decreased ([Painold et al., 2018](#)) but this depended on the specific genus. *Phascolarctobacterium* was negatively associated with three stress measures, thus corroborating some human studies ([Hantsoo et al., 2018](#); [Li et al., 2016](#); [Zheng et al., 2016](#)). *Roseburia* was positively associated with two stress measures, which corroborates some human studies ([Chen et al., 2018b](#); [Jiang et al., 2015](#)) while contradicting others ([Li et al., 2016](#); [Zheng et al., 2016](#)) that also indicate fecal *Roseburia* as a source of beneficial butyrate. *Lachnospiraceae NK4A136* was negatively related to two stress measures. Although the family of

Lachnospiraceae in fecal samples has indeed been negatively related to human depression (Naseribafrouei et al., 2014; Zheng et al., 2016), we could not find reports of this specific genus in the stress literature. In general this family is known for the health beneficial butyrate and general short chain fatty acid production as also observed in children (Dong et al., 2018). The same fact of no current report in stress research was true for fecal *Christensenellaceae*, *Tenericutes uncultured* and *Eubacterium coprostanoligenes*, which showed inconsistent associations with stress. Finally, a positive association with two of our stress parameters was seen for *Rhodococcus* and *Methanobrevibacter*, fecal genera which are not really cited in stress research yet. Fecal *Rhodococcus* has been linked to human gut dysbiosis like ulcerative colitis (Sasaki and Klapproth, 2012). Fecal *Methanobrevibacter* is mainly known as methane producing and energy-efficient genus that is increased in people with anorexia while decreased in overweight (Million et al., 2013).

#### 4.3. Associations with stress reports

Happiness was the stress measure which exhibited the strongest confounder adjusted association with gut microbiota. Still, all questionnaires resulted in 15 to 24 significant OTU associations. The finding that negative emotions are less relevant corroborates the findings of two other studies in healthy people. In 91 healthy women, fecal microbiota status was not at all associated with anxiety, depression or perceived stress (Kleiman et al., 2017). In three people in a closed environment, anxiety, anger and depressive mood seemed less related to fecal bacterial changes over time than vigor (as a positive state) and confusion (as a negative state) (Li et al., 2016). We can thus confirm the importance of positive emotions/mood. Nevertheless, there might be differences depending on the psychological report as a probiotic study found decreases in anxiety and depression but not in perceived stress (Messaoudi et al., 2011). Probiotic intake has been shown to control emotion processing as measured by lab tests on cognitive reactivity to sad mood (using the validated 'revised Leiden index of depression sensitivity scale') and functional magnetic resonance imaging in mid-brain, insula and somatosensory cortex on brain connectivity and emotional attention task responsivity in healthy individuals (Steenbergen et al., 2015; Tillisch et al., 2013), with a higher abundance of fecal *Prevotella* in particular being related to worse emotion regulation and thus increased emotional arousal (Tillisch et al., 2017). In our sample, *Prevotella* was related to both positive and negative emotions but in an inconsistent direction. Positive mood has in one study been associated with fecal *Roseburia*, *Phascolarctobacterium*, *Lachnospira*, and *Prevotella* while negative mood with *Faecalibacterium*, *Bifidobacterium*, *Bacteroides*, *Parabacteroides* and *Anaerostipes* and inter-individual opposite findings for *Lachnospiraceae uncultured* and *Roseburia* (Li et al., 2016). We could confirm the positive relation of *Bifidobacterium*, *Bacteroides* and *Parabacteroides* with negative emotions and the negative relation of *Phascolarctobacterium* while *Prevotella* showed inconsistent associations and high *Roseburia* levels were associated with more stress.

#### 4.4. Associations with cortisol

To our knowledge, no studies exist that document the relationship between hair cortisol as a marker of long-term stress exposure and microbiota composition as measures have been limited to blood/urine/saliva measures of cortisol that are less representative of chronic stress exposure. Probiotic interventions have shown a decrease in salivary or urinary cortisol in humans at baseline (Kato-Kataoka et al., 2016; Messaoudi et al., 2011; Schmidt et al., 2015; Takada et al., 2016), after stress-induction (Allen et al., 2016) and blood corticosterone in rodents (Bravo et al., 2011; Sudo et al., 2004). Nevertheless, there are also a few non-significant findings for probiotics with *Lactobacillus* species on human plasma cortisol levels (Rudzki et al., 2018) and the salivary cortisol response to a lab stressor (Kelly et al., 2017). Similarly,

transplantation of a depression-associated human microbiota to rodents did not change their plasma corticosterone (Kelly et al., 2016). Although saliva cortisol and stress reports during pregnancy did not correlate with each other, both were related to infant gut microbiota: infants of mothers with high cortisol/stress values had higher abundance of some Proteobacteria while lower abundance of lactic acid bacteria and Bifidobacteria (Zijlmans et al., 2015). We could only find one study that reported direct associations between human blood cortisol levels and specific taxa: the cortisol response to stress in pregnant women was positively related with abundance of *Rikenellaceae* and *Dialister* and negatively with *Bacteroides* (Hantsoo et al., 2018). In our population, hair cortisol was only related to a higher relative abundance of *Ruminococcaceae UCG014*.

#### 4.5. Associations with HRV

Some rodent studies showed that the effect of probiotics on anxiety/depression was dependent on the nervous vagus as vagotomization nullified the behavioral and physiological results (Bercik et al., 2011; Bravo et al., 2011; Takada et al., 2016). However, probiotic effects are very strain-specific and vagus-independent pathways also exist since vagotomy failed to block certain signals. Certain bacteria like *Lactobacillus*, *Escherichia* and *Bacillus* can produce neurotransmitters like norepinephrine and acetylcholine (Cryan and Dinan, 2012). The nervous vagus can sense metabolites (direct bacterial products like short chain fatty acids or indirect by enteroendocrine cell activity) via receptors on its afferent nerves to signal to the brain; in the other direction stress depresses nervous vagus activity with then reduced suppression of intestinal permeability and inflammation. Nevertheless, human observational or interventional studies on depression or stress have not yet considered the role of the parasympathetic nervous system. In our study, the parasympathetic parameter was one of the strongest readouts correlated with microbiota composition.

#### 4.6. Strengths and limitations

This is the first study to describe gut microbial differences depending on stress parameters in children/adolescents. Most research has been done in patients with depression (case-control or intervention) and a few papers on healthy adults exist. The use of hair cortisol and HRV, both reliable physiological stress measures, is a novel feature of this report and important in establishing stress-microbiome associations. In addition, we included positive emotions to confirm the only study that made the distinction between positive and negative mood (Li et al., 2016). We present both raw associations as well as analyses adjusted for important confounders like diet, physical activity, sleep socio-economic status and overweight. Nevertheless, diet factors were retrieved from a food frequency questionnaire, so no information on total energy intake was available.

A first limitation is the cross-sectional nature that withholds any statement of the direction of the relationship (stress influencing microbiota, microbiota influencing stress). Although our sample size is larger than most other stress-microbiota papers (e.g. 48 healthy women), some parameters like cortisol were only available in a subset resulting in less power and much larger populations are necessary in future studies. Large European cohorts have reported high levels of inter-individual variation in microbiota composition and suggest that any individual factor would probably have only a very modest effect size e.g. depression could explain 0.2% of variance while all tested predictors together still explained lower than 20% (Falony et al., 2016; Zhernakova et al., 2016). Another limitation is the representativeness of our population. There was enough variety in stress level as around 25–30% were at risk for stress, but all participants came from the same city with rather high socio-economic status and very low prevalence of overweight individuals. Inter-individual difference in mood-taxa correlations in healthy humans have been shown (Li et al., 2016), thus

illustrating these findings might be inherent to population characteristics. Finally, we used a method of 16S rRNA analysis which does not give a high level of resolution i.e. we cannot distinguish beneficial and harmful taxa as that often requires species-level resolution and we have no information on functional activity of the taxa i.e. metatranscriptomics. Therefore, whole-genome metagenomic shotgun sequencing is recommended. After all, even OTUs from the same genus gave sometimes contradictory results.

## 5. Conclusion

First, our hypothesis was confirmed that in children/adolescents stress parameters are also cross-sectionally related to gut microbial composition independent of dietary intake. Second, a unique finding of this study was that mainly positive emotions (in contrast to negative emotions) and the parasympathetic system (reflected by pnn50) seemed to distinguish different microbial compositions. Thus these measures should be integrated in future microbiota projects to confirm our findings in longitudinal and interventional research. However, these parameters are not yet used in standard psychological/psychiatric clinical practice as their applicability needs to be validated first. Moreover, the use of several questionnaires is recommended as taxa associations were highly instrument specific. Third, many associations were OTU specific. This highlights the complex structural alterations and the sensitivity of certain bacterial groups to stress exposure. Fourth, we could confirm the literature on lower Firmicutes on phylum level, increased *Bacteroides* and *Parabacteroides* with stress and decreased *Phascolarctobacterium* in high stress groups but found some new stress-sensitive taxa in association with high stress like higher *Rhodococcus* and *Methanobrevibacter*, lower *Lachnospiraceae* NK4A136 and mixed findings for several genera.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbi.2019.05.024>.

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