Received: 10 May 2020 | Revised: 19 November 2020 | Accepted: 27 November 2020

DOI: 10.1002/eat.23435

ORIGINAL ARTICLE

International Journal of EATING DISORDERS WILEY

Gut microbiota alteration in adolescent anorexia nervosa does not normalize with short-term weight restoration

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Funding information
European Union and its program: ERA-NET (European Research Area Networks) Neuron, Grant/Award Number: 01EW1906A; European Union's Framework Programme for Research and Innovation Horizon 2020; German Ministry for Education and Research (BMBF)

Action Editor: Guido Frank

Abstract

Objective: Gut microbiota are linked to metabolic function, body weight regulation, and brain and behavioral changes. Alteration of gut microbiota is repeatedly demonstrated in adults with anorexia nervosa (AN) and transplantation of stool from adult patients with AN reduces weight gain, food consumption and food efficiency in germ-free mice. No similar data are available for adolescents, who might differ from adults due to their shorter duration of illness.

Method: Nineteen female adolescent patients with AN at admission and after short-term weight recovery were included in a longitudinal study and compared to 20 healthy controls (HC). DNA was extracted from stool samples and subjected to 16S rRNA gene sequencing and analysis. Group comparisons, indicator genera and simper analysis were applied. Taxon abundances at admission was used to predict inpatient treatment duration.

Results: Alpha diversity is increased in patients with AN after short-term weight recovery, while beta diversity shows clear group differences with HC before and after weight gain. A reduction in *Romboutsia* and taxa belonging to Enterobacteriaceae at both timepoints and an increase in taxa belonging to Lachnospiraceae at discharge are most indicative of patients. Lachnospiraceae abundance at admission helped to predict shorter inpatient treatment duration.

Discussion: This pilot study provides first evidence of gut microbiota alterations in adolescent patients with AN that do not normalize with weight gain. If verified in larger studies, the predictive power of taxa belonging to Lachnospiraceae for clinical outcome could complement known predictors at admission, inform clinicians and serve as a target for nutritional interventions.

KEYWORDS

adolescents, anorexia nervosa, eating disorder, gut-brain axis, inflammation, longitudinal, microbiota

Nina Schulz and Meriem Belheouane are joint first author.

1 | INTRODUCTION

There is a fast growing body of literature demonstrating the important role of gut microbiota in metabolic processes, regulating body weight (Harmsen & de Goffau, 2016; Thaiss, Zmora, Levy, & Elinav, 2016) and influencing brain function and behavior via the gut-brain axis (Cryan et al., 2019). These processes are obviously affected in anorexia nervosa (AN) and thus motivate further exploration of the role of microbiota in the etiology, maintaining factors and weight rehabilitation of AN (Bulik, Flatt, Abbaspour, & Carroll, 2019; Mack et al., 2018).

The gut microbiota consists of bacteria, archaea, viruses, and fungi living in the gut and forming complex communities. While diet is one of the main influencing factors on the gut community, microbiota are in turn an important influencing factor on body weight and vary in patients with obesity, normal weight and underweight (Bai, Hu, & Bruner, 2019). Stool transplantations from obese patients into germ-free mice (gf-mice) can lead to an increase in weight, which is associated with the capability to extract more energy from the same amount of food (Ridaura et al., 2013). Stool from malnourished patients showed the opposite effect. Gut microbiota are implicated not only in inflammatory processes and diseases such as inflammatory bowel diseases, diabetes and arthritis (Bravo-Blas, Wessel, & Milling, 2016; Miyoshi & Chang, 2017; Tilg & Moschen, 2014), but also in psychiatric diseases such as depression and anxiety (Luna & Foster, 2015). Kelly et al. (2016), for example, showed that stool transplantation from depressed patients into gf-mice can transfer a depressive phenotype.

In a first transplantation study with stool from adult patients with AN into gf mice, Hata et al. showed a causal effect of the gut microbiota for at least the maintenance of AN. The offspring of transplanted animals displayed a decrease in body weight gain, food intake and food efficiency ratio (body weight gain/food intake). They also had reduced serotonin concentrations in the brain stem and showed an increase in anxiety and obsessive behavior (Hata et al., 2019).

Six cross-sectional studies exploring the gut microbiota in AN with 9–33 adult patients and comparing AN with HC have been published to date (Armougom, Henry, Vialettes, Raccah, & Raoult, 2009; Borgo et al., 2017; Hanachi et al., 2019; M. Million et al., 2013; Morita et al., 2015; Mörkl et al., 2017), while three longitudinal studies with 3–44 patients have been performed (Kleiman, Watson, et al., 2015; Kleiman et al., 2017; Mack et al., 2016), of which only Mack et al. had a mixed adolescent/adult sample.

The preliminary results of these studies vary considerably (Kleiman, Carroll, Tarantino, & Bulik, 2015; Mack et al., 2018; Seitz, Trinh, & Herpertz-Dahlmann, 2019). Some studies found reduced bacterial diversity in patients with AN (Kleiman, Watson, et al., 2015; Mörkl et al., 2017), while others did not (Borgo et al., 2017; Mack et al., 2016). Several alterations in the taxa abundances were noted during the acute state (Borgo et al., 2017; Kleiman, Watson, et al., 2015; Mack et al., 2016). It is currently unclear how these alterations impact the course of AN and whether they can be potentially used to help predict (short-term) disease outcome. Importantly, both Kleiman and Mack found evidence of significantly altered microbiota after short-term weight recovery in their mostly chronically ill adult and mixed adult/adolescent samples, again emphasizing that altered microbiota are not simply epiphenomena of undernutrition (Kleiman, Watson, et al., 2015; Mack et al., 2016). However, it remains unclear whether this also applies to adolescent patients as they are less often chronically ill, and microbiota alterations may not become as ingrained as those in adults with a generally longer duration of illness.

Finding altered microbiota in patients with AN and especially identifying those associated with a positive outcome could lead to new intervention targets. These taxa could be isolated, cultivated and administered as probiotics or alternatively, nutritional or prebiotics indirectly fostering their growth could be tested to supplement current refeeding practices in AN. There is growing evidence for the effect of these interventions in other psychiatric or "weight regulation" disorders such as obesity or underweight (Depommier et al., 2019; Matthieu Million, Lagier, & Raoult, 2017; Pirbaglou et al., 2016; Trehan et al., 2013). We thus decided to compare gut microbiota diversity and taxon abundances in a longitudinal sample of adolescent patients with AN and compare them to those of age-matched HC. We hypothesized that the microbiota of adolescents with AN—corresponding to the results in adults—differ from that of HC and do not normalize with weight recovery, despite the younger age, shorter illness duration in most cases and age-dependent different hormonal status. We furthermore hypothesized that the abundance of certain taxa at admission could help predict duration of inpatient treatment.

2 | METHOD

2.1 | Patients and controls

Twenty-two consecutive patients were initially recruited at the University Hospital Aachen and enrolled between November 2016 and January 2018. The time points measured were admission to inpatient hospital treatment and discharge to outpatient care on average 4 months later. Three patients were prescribed antibiotics between admission and discharge and were excluded from the analysis, leaving 19 patients to be analyzed. Additionally, 20 age-matched healthy female controls (HC) were enrolled using newspaper advertisements.

The inclusion criteria were as follows: diagnosis of AN according to DSM 5, female sex, and age between 12 and 18 years. The exclusion criteria were as follows: use of antibiotics or probiotics within 4 weeks before enrollment, IQ < 85, insufficient knowledge of German language, severe other mental disorder and severe gastrointestinal or metabolic illnesses such as celiac disease or diabetes mellitus. The same exclusion criteria were also applied for the healthy volunteers in addition to having a high or low bodyweight (>80th or <20th age adjusted percentile of body mass index [BMI-SDS]), any current psychiatric illness and any lifetime eating disorder. All participants and their legal guardians gave written informed consent prior to enrollment. The agreement of the local ethics committee of the University of RWTH Aachen was obtained for this study, and the study was conducted in accordance with the Declaration of Helsinki.

Clinical data including body weight after an overnight fast measured in BMI and BMI-SDS calculated using Ped(z) calculator with KiGGS Data (Schaffrath, 2003) at admission and discharge were noted as well as BMI prior to the illness, weight loss prior to admission, illness duration, weight gain during treatment and the duration of treatment. Any medication use at admission was noted and sorted into the following groups to be each used as a binary covariate: laxatives, antibiotics, antidepressants, gastrointestinal medications, others.

Patients received treatment as usual that included weight rehabilitation, individual and group psychotherapy, parent psychoeducation and training, occupational-, music- and physical therapy. Nutritional therapy started with 1,200 kcal/day and was augmented every second day by 200 kcal/day until weight increased by 500–1,000 g/week up to the 20th–25th age adapted BMI-percentile.

2.2 | Questionnaires and interviews:

Each participant in the study completed three questionnaires at admission and discharge: The Eating Disorder Inventory 2 (EDI), Beck Depression Inventory (BDI) and Spence Children's Anxiety Scale (SCAS) (Beck, Steer, Ball, & Ranieri, 1996; Garner & Olmsted, 1986; Spence, 1998). Patients also received the semi-structured eating disorder interview EDE (Eating disorder Examination, first German edition 2016) (Cooper, Cooper, & Fairburn, 1989; Luce & Crowther, 1999).

2.3 | Fecal sample collection and DNA extraction:

Fecal samples were collected by the patients within 2 weeks of admission and at discharge using a single-use paper stool catcher (The Feces Catcher, Tag Hemi VOF, Netherlands) by transferring a pea-sized sample from two different sites of the stool into a sterile plastic container. The stool samples were stored at −80°C until further use. Time between stool sampling and symptom report was kept to a minimum and averaged 4.1 days.

Healthy volunteers were instructed to collect their stool at home using the same procedure and bring or send the samples to the clinic to be frozen at −80°C until further use. All samples were then sent on dry ice to JB and MB for analysis.

To extract DNA from stool samples, we used the DNeasy Power Soil Kit (Qiagen) following the manufacturer's instructions and matching procedures of the Human Microbiome Project (The Human Microbiome Project Consortium, 2012).

2.4 | 16S rRNA gene sequencing and processing

We sequenced the V1-V2 region of the 16S rRNA gene using primers 27F and 338R consistent with the Human Microbiome Project and previous works of our group (Claussen et al., 2017). We followed a dual barcoding approach whereby forward and reverse primers contained a unique eight base multiplex identifier to tag PCR products.

During demultiplexing, no mismatch in the barcode was allowed (Casava, Illumina). Forward and reverse reads were merged in Usearch (v.7) (Edgar, 2010) as follows: reads were truncated at the first base where the quality score dropped below Q = 3, the maximum number of mismatches in the overlap region was 2, the minimum length of reads after truncation was 200 bp, the minimum length of the overlap region was 150 bp, the minimum length of the merged read was 270 bp, and the maximum length of the merged read was 330 bp. Next, merged reads were filtered using the expected error parameter $E = 0.1$. Chimeric sequences were removed in Uchime using the Silva Gold reference database (Edgar, Haas, Clemente, Quince, & Knight, 2011).

RDP Multi-Classifier version (v.9.0) (Wang, Garrity, Tiedje, & Cole, 2007), as implemented in Mothur (v.31) (Schloss et al., 2009), was used to assign taxonomy using a confidence threshold of 0.80 and 1,000 iterations. Sequences classified as eukaryotic, unknown, or mitochondrial were removed. Sequences were aligned to the Silva reference database (Pruesse et al., 2007), and those that did not align were removed. Aligned sequences were denoised using the "pre-cluster" algorithm in Mothur. Prior to OTU binning, sequences were rarefied to 10,000 reads per sample. OTUs were binned at a 97% similarity threshold using distance-based greedy clustering (dgc) implemented in Mothur.

2.5 | Statistics

2.5.1 | Group comparisons of microbiota data

All statistical analyses were carried out in R (v.3.6.1) (R Core Team, 2019). Comparison of core taxon abundances (defined as present in at least 25% of the individuals, with at least 1% relative abundance) was performed using the Mann–Whitney U and Wilcoxon signed rank tests, and p values corrected for multiple testing according to Benjamini and Hochberg (1995). As effect size measure, we report r (see Mangiafico, 2016), which is defined as the Z value from the test divided by the total number of observations (Mann–Whitney U-test) or by the number of samples (Wilcoxon signed Rank test), on a scale of 0 to 1, with values of >0.5 usually considered a large effect.

To identify taxa that contribute to the disparities in microbiota composition across treatment groups (patients at admission $n = 19$, patients at discharge $n = 19$, and healthy controls $n = 20$), we performed (a) indicator taxa analyses in the "indicspecies" R package (De Cáceres & Legendre, 2009) using the "r.g" function (Cáceres, Legendre, & Moretti, 2010) and 10^5 permutations and (b) "simper" analysis in the "vegan" R package (v.1.7.9) (Oksanen, 2007) with 10^5 permutations. For the indicator taxa analysis, the "effect size" parameter indicates the strength of the association on a scale of 0 to 1 (parameter "stat" of r.g function), with higher values indicating a stronger association. For the simper analysis, the reported "contribution" value is an estimator of each genus' contribution to discriminating the groups (parameter "average" of simper function) and therefore can be used to quantify the strength of the effect, again, with higher values indicating a stronger contribution to discrimination.

Alpha diversity indices (within sample diversity) were calculated based on the lower OTU-species level with the scope of revealing potential disparities in distribution patterns across treatment groups that we might not detect at the phylum and genus levels using "diversity" and "estimateR" in the "vegan" (v.2.5-6) R package and compared across individual groups as mentioned above. Additionally, we assessed individual diversity (beta diversity) at the OTU-species level using Bray-Curtis and Jaccard indices, which are based on disparities in taxon abundances and presence/absence, respectively, and evaluated the influence of the individual groups using PERMANOVA "adonis" and constrained principal coordinates analyses with 10^5 permutations in "vegan."

To assess associations with clinical parameters, we calculated Spearman correlations between alpha-diversity at admission, discharge and the difference between both time-points with respective values of BMI-SDS, BDI, SCAS, and EDE as well as with illness duration and prior weight loss.

2.5.2 | Prediction analysis: Effect of the gut microbiota at admission on the duration of treatment

The duration of treatment and not BMI-SDS at discharge was selected as the clinical outcome parameter, due to the target weight being fixed at the 20th to 25th age-adjusted BMI percentile and thus containing only limited variance. To control for known predictors of clinical outcome and inpatient treatment duration, we first constructed a linear model with duration of treatment as the dependent variable and weight loss prior to treatment (BMI-SDS), illness duration and absolute weight at admission (BMI-SDS) as explanatory variables. Next, we built a second linear model that included the residuals of the first linear model as the dependent variables and the relative abundance of the microbial trait as an explanatory variable for each of the core genera, together with medication use, with the different medication groups as binary covariates.

To check the models, we (a) inspected the distribution of the residuals, (b) plotted the residual versus fitted values, and (c) examined the residual distribution across the explanatory variables included in the models. If needed, square root transformation of microbial relative abundances was used to correct for non-normal distribution.

3 | RESULTS

For the clinical characterization of patients at admission and discharge, please see Table 1.

TABLE 1 Clinical sample characteristics

	AN admission *n* = 19	AN discharge *n* = 19	HC *n* = 20
Age (years)	15.77 (1.94) [12.01;18.63]	16.16 (1.99) [12.24;18.99]	16.35 (1.11) [14.31;17.89]
BMI (kg/m^2)	15.76 (2.03) [13.04;18.88]	18.8 (0.87) [16.84;20.11]	20.31 (2 .35) [17.04;25.32]
%EBW	75.13 (10.25) [60.1;91.58]	79.82 (3.9) [79.82;95.83]	94.77 (10.31) [81.34;116.66]
BMI-SDS (z-score)	−3.08 (2.07) [−6.78;-0.59]	−0.97 (0.42) [−2.16;0.28]	−0.51 (0.82) [−1.86;0.94]
BMI prior to admission (kg/m^2)	20,2 (2,72) [15,5;26,6]		
Premorbid body weight (BMI)	4.44 (3.09) [1;13.09]		
Weight loss prior to admission (BMI-SDS)	2.73 (2.02) [0.61;7.52]		
Illness duration (month)	17.16 (16.12) [3;62]		
Weight gain during therapy (BMI)		3.17 (2.07) [0.77;7.84]	
Weight gain during therapy (BMI-SDS)		2.11 (1.92) [0.31;5.47]	
Therapy duration (month)		4.05 (1.39) [2;7]	
EDI 2 total score	295.16 (50.69) [168;372]	270.26 (68.28) [146;415]	183.15 (36.59) [123;236]
BDI 2 score	22.68 (19.95) [0;41]	17.00 (15.75) [0;54]	5.65 (4.87) [0;17]
SCAS total score	28.271 (14.79) [2;67]	19.74 (17.08) [0;75]	17.85 (9.61) [4;39]
EDE total score	4.50 (1.19) [1.35;5.76]	2.82 (1.49) [0.43;5.38]	
Atypical AN	*N* = 2 (10.5%)		
Binge/purge subtype	*N* = 1 (5.3%)		

Note: Values are depicted as the mean, standard deviation in brackets and minimum and maximum values in square brackets. *p*-values are calculated between AN at admission versus AN at discharge.

Abbreviations: AN, Anorexia Nervosa; BDI, Beck Depression Inventory; BMI-SDS, Body mass index—standard deviation scores; EDE, Eating Disorder Examination; EDI, Eating Disorder Inventory; HC, Healthy Controls; SCAS, Spence Children's Anxiety Scale; %EBW: Percent expected body weight.

3.1 | Differences in core phylum abundances between patients and HC

When inspecting the abundances of the core phyla (Figure 1a and Figure S1a), we find Firmicutes to significantly increase with weight gain (adjusted *p*-value .002, effect size *r* = 0.69) to a significantly higher level in patients at discharge versus HC (adjusted *p*-value .0005, effect size *r* = 0.56; see Table 2A for results on all core phyla).

3.2 | Patterns of core genera across patients and HC

First, in pairwise comparisons we found *Anaerostipes* to be increased in patients at admission versus HC, while *Romboutsia* and on a trend level unclassified Enterobacteriaceae are reduced (adjusted *p*-values: .012, .008, and .058, effect sizes *r* = 0.46, 0.48, and 0.38, respectively; Figure 1b). Significant increases following weight gain are found for *Fusicatenibacter*, *Lachnospiracea*, *Ruminococcaceae*, and *Faecalibacterium* while *Bacteroides* decrease between admission and discharge (adjusted *p*-values .022, .0008, .007, .032; .001, *r* = 0.39–0.74). At discharge, *Romboutsia* and on the trend level unclassified Enterobacteriaceae remain decreased in patients, while *Fusicatenibacter* and unclassified Lachnospiraceae are newly increased in patients compared to HC (adjusted *p*-values: .044, .059, .022, and 3.50×10^{-5}, *r* = 0.33–0.71, see Table 2B for results on all core genera). Second, we performed indicator taxa analyses, which examines the affinity or preference of a given taxon to one- or a combination of defined environments. These analyses reveal unclassified Enterobacteriaceae and *Romboutsia* to be associated to healthy controls (effect size = 0.40, *p* = .03; effect size = 0.33, *p* = .09, respectively), while unclassified Lachnospiraceae is strongly associated with patients at discharge (effect size = 0.61, *p* = .0001; Figure 1c, Table S1).

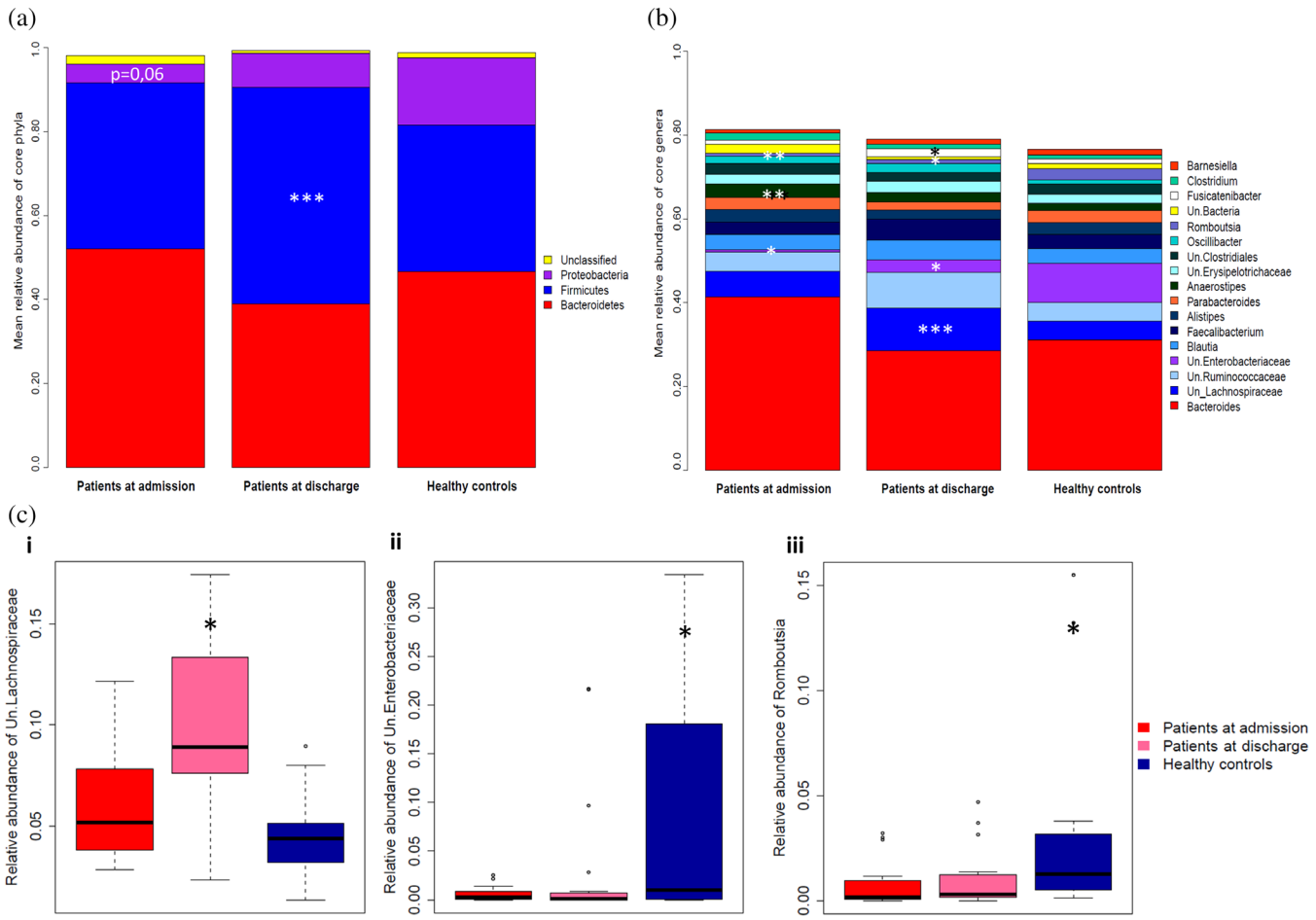

FIGURE 1 Mean relative abundance of core phyla (a), and genera (b) and indicator genera (c) in disease and healthy states. Samples from patients with AN at hospital admission (*n* = 19) and discharge (*n* = 19) and HC (*n* = 20) at the (a) phylum and (b) genus taxonomic levels. Core samples were defined as present in at least 25% of the individuals with at least 1% relative abundance. Comparisons between AN and HC: Mann–Whitney *U* test with $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. Longitudinal comparisons Wilcoxon signed rank test with $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$. (c) Box plots of the relative abundance of significant indicator genera defining (i) patients at discharge or (ii-iii-C) typical for HC (i) Un. Lachnospiraceae, (ii) Un. Enterobacteriaceae and (iii) *Romboutsia*. $^{*}p < .05$ AN, anorexia nervosa; HC, healthy controls [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Mean relative abundances, Mann Whitney-U, Wilcoxon Rank test and effect size of phylae and genera

	Healthy controls	AN patients ad-mission	AN patients dis-charge	AN admission versus HC corr. *p*-value Effect size r	AN discharge versus HC corr. *p*-value Effect size r	AN admission versus AN discharge *p*-value Effect size r
Panel A: *Phylum*						
Bacteroidetes	46.70	52.03	39.03	.34	.13	**.044**
				r = 0.155	*r* = 0.274	***r* = 0.564**
Firmicutes	34.91	39.58	51.43	.15	**.0005**	**.002**
				r = 0.234	***r* = 0.576**	***r* = 0.693**
Proteobacteria	15.98	4.48	8.21	.09	.13	.86
				r = 0.346	*r* = 0.274	*r* = 0.0461
Unclassified	1.22	2.05	0.68	.83	.33	.099
				r = 0.036	*r* = 0.199	*r* = 0.493
Panel B: *Genus*						
Bacteroides	31.19	41.44	28.54	.19	.55	**.0013**
				r = 0.248	*r* = 0.099	***r* = 0.748**
Unclassified Enterobacteriaceae	9.27	0.58	3.05	.059	.06	.87
				r = 0.376	*r* = 0.331	*r* = 0.0369
Un. Ruminococcaceae	4.53	4.53	8.42	.9	.12	**.0072**
				r = 0.0226	*r* = 0.282	***r* = 0.665**
Un. Lachnospiraceae	4.38	6.09	10.19	.13	**.00004**	**.0008**
				r = 0.248	***r* = 0.705**	***r* = 0.739**
Blautia	3.51	3.63	4.74	.43	.1	.1
				r = 0.128	*r* = 0.315	*r* = 0.415
Faecalibacterium	3.37	2.96	4.93	.46	.46	**.032**
				r = 0.122	*r* = 0.153	***r* = 0.574**
Alistipes	2.86	2.95	2.20	.52	.48	.47
				r = 0.106	*r* = 0.162	*r* = 0.333
Parabacteroides	2.78	2.83	1.85	.47	.14	.12
				r = 0.119	*r* = 0.271	*r* = 0.484
Romboutsia	2.77	0.73	0.97	**.008**	**.044**	.4
				***r* = 0.482**	***r* = 0.351**	*r* = 0.203
Un. Clostridiales	2.32	2.61	2.02	.97	.97	.97
				r = 0.009	*r* = 0.0179	*r* = 0.0369
Un. Erysipelotrichaceae	2.17	2.28	2.61	.76	.76	.76
				r = 0.0562	*r* = 0.171	*r* = 0.0739
Anaerostipes	1.83	3.28	2.36	**.012**	.19	.19
				***r* = 0.461**	*r* = 0.213	*r* = 0.351
Barnesiella	1.40	0.81	1.26.	.97	.97	.77
				r = 0.0701	*r* = 0.00908	*r* = 0.266
Un. Bacteria	1.22	2.05	0.68	.83	.33	.099
				r = 0.036	*r* = 0.1999	*r* = 0.493
Fusicatenibacter	1.05	1.01	1.83	.45	**.023**	**.023**
				r = 0.124	***r* = 0.388**	***r* = 0.583**
Oscillibacter	0.99	1.71	2.19	.36	.36	.98
				r = 0.216	*r* = 0.191	*r* = 0.00461

TABLE 2 (Continued)

	Healthy controls	AN patients ad-mission	AN patients dis-charge	AN admission versus HC corr. *p*-value Effect size r	AN discharge versus HC corr. *p*-value Effect size r	AN admission versus AN discharge *p*-value Effect size r
Un.Clostridium sensu_stricto	0.87	1.75	1.09	.91 *r* = 0.0202	.52 *r* = 0.153	.45 *r* = 0.335

Note: Results of Mann–Whitney-*U*, Wilcoxon signed rank tests and effect sizes between patients with AN and HC of all (A) core phylae and (B) core genera. Core phylae and genera were defined as being present in at least 25% of the sample with at least 1% relative abundance. Abundances of healthy controls, patients at admission and patients at discharge and corresponding corrected *p*-values are shown. Significant effects ($p < .05$) are marked in bold. Effect size calculation: As effect size measure, we report *r* (see for example, Mangiafico, 2016), which is defined as the *Z* value from the test divided by the total number of observations (Mann-Whitney *U*-test) or by the number of samples (Wilcoxon signed Rank test), on a scale of 0 to 1, with values of >.5 usually considered high effect sizes.
Abbreviations: Un., unclassified; corr., corrected (Benjamini & Hochberg, 1995).

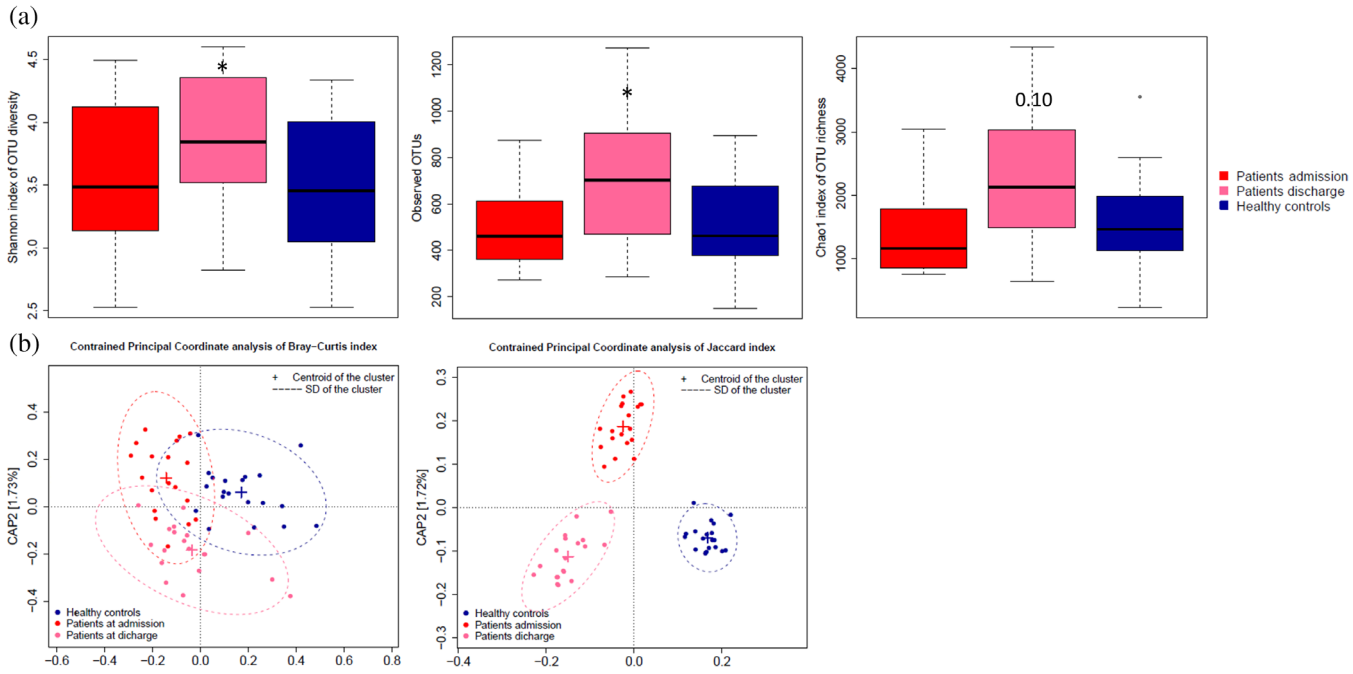

FIGURE 2 Alpha and beta diversity measures. Alpha and beta diversity based on species-level OTUs in samples from patients with AN at hospital admission ($n = 19$) and discharge ($n = 19$) and HC ($n = 20$). (a) Alpha diversity (richness) was characterized by the number of observed bacterial species in each sample, the Shannon index and the Chao1 estimator of diversity. Differences in alpha diversity were calculated using the two-sided Wilcoxon pairwise run sum test. (b) Beta diversity was assessed by the Bray-Curtis and Jaccard indices and tested using PERMANOVA "adonis" and constrained principal coordinates analyses with 10^5 permutations in "vegan". Comparisons between AN and HC: Mann–Whitney *U* test with $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. Longitudinal comparisons Wilcoxon signed rank test with $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$. AN, anorexia nervosa; HC, healthy controls; OTU, operational taxonomic units [Color figure can be viewed at wileyonlinelibrary.com]

Finally, we applied a "simper" analysis to identify taxa that discriminate between patients and HC. We find that *Anaerostipes* significantly contributes to differentiating patients at admission and HC (contribution = 2%, $p = .025$), whereas the abundances of unclassified Lachnospiraceae, unclassified Enterobacteriaceae, unclassified Ruminococcaceae and *Barnesiella* differentiate between patients at discharge and HC (4%, 6%, 4%, $p < .05$, respectively; Figure S2 and Table S2).

3.3 | Alpha and beta diversity

We observe no difference between patients with AN at admission and HC in any of the alpha-diversity measures calculated on the most sensitive OTU level, which measures diversity within subjects. However, during weight gain, alpha diversity increases significantly and when comparing AN at discharge with HC, we find a significantly increased total number of species and Shannon

index in the patient group (p = .028 and p = .035, respectively, Figure 2a).

The (between groups) beta diversity comparison on the OTU level reveals significant differences between patients and HC based on the Bray-Curtis and Jaccard indices (Bray-Curtis, PERMANOVA, R^2 = 0.05, p = .02, constrained principal coordinate analyses, constrained inertia = 0.05, p = .02; Jaccard, PERMANOVA, R^2 = 0.04, p = .007, constrained principal coordinate analyses, constrained inertia = 0.04, p = .005). These differences do not normalize upon weight gain, showing clear differences between the community structures of patients with AN at discharge (Figure 2b).

We found no correlations between alpha diversity measures and clinical parameters.

3.4 | Predictive effect of the gut microbiota at admission on the duration of treatment

To investigate the potential influence of individual core taxon abundances in patients at admission on the duration of treatment, we used a linear model approach to first correct for the influence of duration of illness, prior weight loss and BMI-SDS at admission. This yielded a model explaining 18% of the variance in duration of treatment (F-statistic: 2.33 on 3 and 15 DF, p = .11, R^2 = 18%).

Next, we evaluated each of the core genera for an added predictive effect. We find that a higher abundance of unclassified Lachnospiraceae in patients at admission is associated with a shorter duration of treatment (F-statistic: 4.86 on 1 and 17 DF, p-value: .04, Beta coefficient value: −0.90), explaining an additional 18% of the variance of duration of treatment (Figure 3). Interestingly, GI medication also displays a trend-level significant effect on predicting the duration of treatment (F-statistic: 3.25 on 1 and 17 DF, p-value = .089), explaining 11% of the variance. Jointly with GI tract medication, Lachnospiraceae explains 21% of the residual variation in inpatient treatment duration (whole model: F-statistic, 3.43 on 2 and 16 DF, p = .05; Lachnospiraceae, beta coefficient = −0.74, p = .03, GI-medication beta coefficient = −0.086, p = .08), thus correcting for GI medication use at admission.

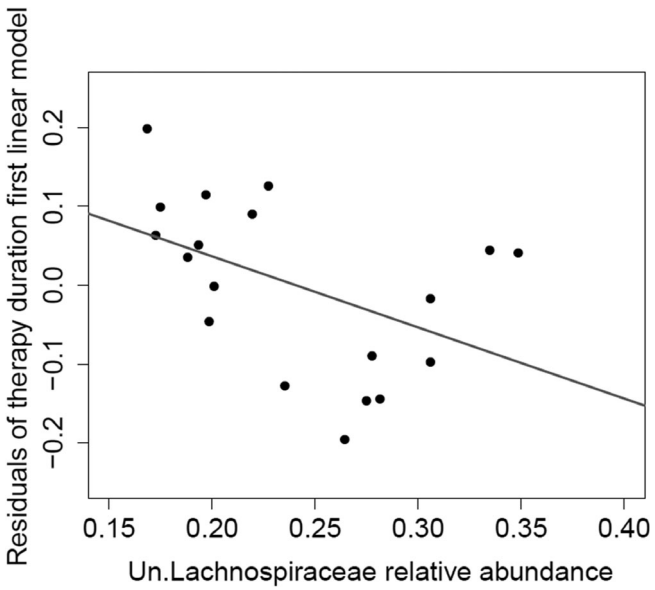

FIGURE 3 Lachnospiracea predicting inpatient treatment duration. Results of the second linear model including relative abundance of unclassified Lachnospiraceae predicting inpatient treatment duration corrected for duration of illness, prior weight loss and low BMI-SDS at admission. F-statistic, 4.86 on 1 and 17 DF; p-value, .04; Beta coefficient value, −0.90

4 | DISCUSSION

The goal of this study was to provide a first assessment of the gut microbiota in adolescents with AN, given the differences previously observed in adults with the disease. We further examined whether the differences between AN versus HC adolescents revert after weight recovery, and whether microbiota help to predict the duration of treatment. Our adolescent patients indeed display marked differences compared to HC in this pilot study. Further, weight-recovered patients still harbor distinct differences in their gut microbiota, supporting the hypothesis that our observations in adolescent AN are more than just a weight-related epiphenomenon, despite a shorter duration of illness of on average 17 months. Interestingly, this dysbiosis in adolescents appears to be different compared to that in adults regarding overall community patterns and the types of individual taxa involved. Furthermore, we find a higher abundance of taxa belonging to Lachnospiraceae to predict a shorter inpatient treatment duration independent of illness duration, prior weight loss and absolute low weight at admission.

The gut microbial patterns in adolescent AN at admission are characterized in the indicator analysis and group comparison by a reduction in *Romboutsia* and taxa belonging to Enterobacteriaceae, which remain present after weight recovery, following on average 4 months of treatment. Further, unclassified Lachnospiraceae significantly increased to higher levels than that of HC, despite weight rehabilitation. Additionally, the absolute number of species found in patients at discharge was significantly higher than that of HC. Thus, similar to adults with AN (Kleiman et al., 2017; Kleiman, Watson, et al., 2015; Mack et al., 2016), change of the microbiota in adolescents with AN is not simply a function of weight loss and does not disappear upon (short-term) weight restoration. This is compatible with the notion that, comparable to adults, microbiota could play an important role in the pathophysiology of AN. To further test this hypothesis, longer-term follow-up studies are needed in adolescent (and adult) patients. Future studies should analyze whether these differences after short-term weight recovery only occur during a temporary adjustment period, are associated with still-altered feeding behavior commonly found in AN (Tomba, Tecuta, Crocetti, Squarcio, & Tomei, 2019) or persist after long-term recovery and are thus are trait markers of the disease.

Interestingly, there are also several differences in adolescent - compared to adult patients with AN, especially regarding the

intraindividual alpha diversity and the specific phyla and genera involved. While the overall number of species in prior studies of adult patients with AN was found to be either reduced (Kleiman, Watson, et al., 2015; Mörkl et al., 2017) or unaltered (Hanachi et al., 2019; Mack et al., 2016), we did not find any alterations in adolescents at admission. In contrast, after weight recovery, we even found an increased alpha diversity, a finding that was only shared by Mack et al., the single previous study involving a mixed sample of both 44 adults and 11 adolescents (Mack et al., 2016). This increase could reflect a temporary adjustment to weight rehabilitation, be nutrition-related or represent a specific marker in adolescent AN A similar picture was observed among genera. *Anaerostipes* (family of Lachnospiraceae) were significantly increased at admission compared to HC and non-significantly increased at discharge by 50%. This contrasted with the finding that the abundance of *Anaerostipes* was reduced in adults with AN (Hanachi et al., 2019; Kleiman, Watson, et al., 2015) but fits well with the finding of Mack et al. in their sample also comprising 11 adolescents, who found that the abundance of *Anaerostipes* significantly increased both before and after weight rehabilitation. Decreased *Romboutsia* and unclassified Enterobacteriaceae at admission and discharge, while underscoring persistent differences in adolescent AN even after weight recovery, contrasts to the finding of Borgo et al., who observed increased Enterobacteriaceae at admission in adults. Moreover, to our knowledge the increase in unclassified Lachnospiraceae and *Fusicatenibacter* at discharge found in our study was not observed in any adult studies to date. Differences in the AN-associated gut microbiota between adolescents and adults could be caused by multiple factors. While the gut microbiota generally changes with age, this takes place most notably at the beginning and end of life; birth mode, breast feeding and early nutrition play important roles at the beginning of life, and chronic illnesses and medication use as well as nutrition and physical activity play important roles at the end (Galazzo et al., 2020; Takagi et al., 2019). However, adolescents still undergoing development, especially due to their hormonal changes during puberty, do show some age-specific alterations in their gut microbiota compared to adults (Farzi, Fröhlich, & Holzer, 2018). Additionally, adolescents differ from adults in their feeding patterns, physical activity and number of past infections and medication use, all known to have important effects on the gut microbiota.

Mechanisms of the gut microbiota's interaction with the host in AN include less energy harvest from the same food, making weight rehabilitation even more difficult (Aurigemma, Koltun, VanEvery, Rogers, & De Souza, 2018; Seitz, Belheouane, et al., 2019). Microbiota also exert regulatory effects on gut wall permeability (Karl et al., 2017). Higher gut permeability can allow more bacteria and their products to traverse the gut wall and enter cells and the bloodstream, leading to inflammation and (auto-) immune processes (Nagpal & Yadav, 2017). Interestingly, a higher rate of autoimmune diseases is found in AN, as well as low-grade inflammation particularly in the GI tract, including an up to fourfold increased risk of inflammatory bowel diseases, the causes of which remain poorly understood (Hedman et al., 2019; Raevuori et al., 2014). These observations are hypothesized to be associated with increased intestinal permeability in AN (Jésus et al., 2014; Seitz, Belheouane, et al., 2019), but more research is needed. Inflammation is also linked to anxiety, obsessiveness and depression (Li et al., 2019), the three major comorbidities in patients with AN (Herpertz-Dahlmann, Seitz, & Baines, 2017). We did not find any significant associations with anxiety, depressiveness or eating disorder pathology in our sample, potentially due to the small sample size. To disentangle initiating factors from maintaining factors, as well as the effects of illness duration from those of age, transplantation studies into gf-rodents need to be undertaken with adolescent (and adult) donors and illness durations.

Special interest lies in the potential for microbial traits to help predict short-term clinical outcome. We found yet unclassified taxa belonging to Lachnospiraceae to be an interesting candidate, as an increase in their abundance at admission helped to predict a shorter inpatient treatment duration, which is generally associated with a better long-term outcome (Grimaldi et al., 2018). This finding is independent of other known predictors of shorter inpatient treatment duration, such as prior weight loss, illness duration and low body weight at admission. Lachnospiraceae, belonging to the phylum Firmicutes, is one of the most common families in the human gut and is known to decompose otherwise indigestible parts of our food. Thus, it could conceivably play a role in increasing energy utilization in patients with AN. In fact, Lachnospiraceae were shown to be associated with weight gain following antibiotic treatment (Liu et al., 2019) and to increase blood glucose levels (Kameyama & Itoh, 2014). A decrease in Lachnospiraceae is also a hallmark of several inflammatory disorders (Geirnaert et al., 2017; Maukonen et al., 2015) and many Lachnospiraceae species produce butyrate (Vital, Karch, & Pieper, 2017), which is crucial to maintaining intestinal barrier function (Bordoni et al., 2019) and helping to limit the colonic inflammatory response (Lobionda, Sittipo, Kwon, & Lee, 2019). As noted, patients with AN show chronic low-grade inflammation of unknown origin (Dalton et al., 2018) and an increased rate of autoimmune diseases (Hedman et al., 2019; Raevuori et al., 2014). Thus, the anti-inflammatory effects of Lachnospiraceae might also play a role in its association with shorter inpatient treatment duration. Specific taxa belonging to Lachnospiraceae associated with a positive outcome need to be identified, extracted and cultivated to allow for supplementation studies (first in animals). This would help to further elucidate the underlying pathomechanisms and the causality of their impact on weight, inflammation, and behavior. The general potential of microbiota to help to increase weight gain has already been shown several times, for example, in stool transfer studies into mice (Ridaura et al., 2013; Tremaroli et al., 2015). As already mentioned above, *Lactobacillus* and *Bifidobacterium* probiotics have shown an effect regarding anxiety or depression (Pirbaglou et al., 2016), and omega-3 fatty acids have an impact on gut microbiota, gut wall and the immune system (Costantini, Molinari, Farinon, & Merendino, 2017).

Limitations: First, our sample size was small, despite this study being the second largest longitudinal study to date. Nevertheless, our

results need to be validated in larger studies. Second, HC data was not assessed at follow-up to control for a change of diet or other environmental factors. Third, we did not control for the type of diet, although this is known as an important influential factor including the inherently larger quantities consumed by patients at discharge compared to admission. Fourth, differences in analysis protocols, such as the choice of 16S rRNA gene primers (e.g., we used V1-V2 compared to others that used V3-V4), can introduce biases in some individual bacterial taxa, which may in part explain the heterogeneity in findings between studies. However, by comparing the microbiomes across different animal taxa, recent studies also demonstrated that biological variables, such as the host species, prevail over such technical aspects when the overall pattern of beta diversity is considered (Rausch et al., 2019). Fifth, samples could be only acquired during a time span of the first 2 weeks, because it took time to convince the participants and to obtain third party consent. Moreover, many patients had a chronic obstipation and a reduced stool production. Thus, some changes in the microbiota might have occurred. However, patients gained on average only 0.636 kg until sampling, so we expect our analysis to still reflect most effects of being underweight.

5 | CONCLUSION

This is the first study of the gut microbiota in a purely adolescent sample of patients with AN.

In this pilot study, we confirmed a marked alteration of microbiota that persisted after weight recovery. These findings fit well with adult findings and support an important role of microbial alterations also in adolescents, despite their shorter duration of illness. However, the microbial patterns present in adolescent patients appeared somewhat different to those in adults, especially regarding diversity and the specific taxa involved. Causal relations to weight gain and psychiatric symptoms as shown in the adult patient group, still need to be established in adolescents. Taxa belonging to Lachnospiraceae help predict shorter inpatient treatment duration and could be an interesting candidate for future supplementation.

ACKNOWLEDGMENTS

The study was partly funded by the European Union (ERA-NET Neuron, 01EW1906A) "Microbiome Gut Brain Axis in Anorexia Nervosa" project sponsored by the European Union (EU) and the German Ministry for Education and Research (BMBF).

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Schulz N, Belheouane M, Dahmen B, et al. Gut microbiota alteration in adolescent anorexia nervosa does not normalize with short-term weight restoration. *Int J Eat Disord*. 2020;1–12. https://doi.org/10.1002/eat.23435